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# Chemical Characterization of *Cynara Cardunculus* var. *Scolymus* and Its Application in Topical Formulations

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# Abstract

*Cynara Cardunculus* var. *Scolymus*, usually known as artichoke, is a Mediterranean specie with therapeutic properties, including antioxidant activity. This plant is a rich source of polyphenols, including caffeoylquinic and dicaffeoylquinic acids. The use of bioactive ingredients or phytochemicals extracted from plant tissues in cosmetics is increasing, thus artichoke extract due to its constituents can be incorporated in topical formulations.

The artichoke extract used in this study was obtained through an infusion technique, a conventional method in which dried leaves are placed in boiling water for a short period of time. Aqueous artichoke extract was characterized by high-performance liquid chromatography–diode array detector and a quantification of several constituents present in it, like proteins, carbohydrates, polyphenols and dietary fibers, was performed. Two purification strategies were adopted, including a gastric digestion followed by a dialysis that resulted in artichoke fraction A and a mucilage precipitation method. In this last method, two different assays were performed, one using acetic acid and the other without the use of acetic acid, resulting in artichoke fractions B and C, respectively. The purify fraction with higher polyphenols content was artichoke fraction C, which was chosen among all. The content of total phenols and tannins were determined along the purification processes, as well as the antioxidant activity.

Artichoke Extract and Artichoke Fraction C were subjected to ROS scavenging activity and MTT cytotoxicity assays in HaCaT cell lines, being both good antioxidants and non-toxic. Sun protection factor (SPF) was also measured in both fractions and the artichoke extract showed the highest SPF.

These fractions were incorporated in O/W emulsions and hydrogels for topical application. Chemical and physical characterization and microbiological control, as well as cytotoxicity and membrane integrity assays, were performed on the products to ensure their quality and safety.

The formulations containing the artichoke extract were considered the best choice according with the *in vitro* studies realized and the costs and time of production of each fraction, so these were chosen to proceed with the study.

ROS scavenging activity assays of the formulations containing artichoke extract showed powerful antioxidant activities. *In vivo* studies, including a human repeat insult patch testing (HRIPT) and an assessment of the protective effect against oxidative stress after UV radiation by chromameter evaluation, showed very good skin compatibility and no allergenic potential for all formulations, and according to the antioxidant activity assay, the artichoke gel was the formulation that presented a true *in vivo* antioxidant activity.

The aim of this study was to investigate the antioxidant properties of artichoke extract in topical formulations.

Key words: *Cynara Cardunculus* var. *Scolymus*; artichoke; purification strategies; phenols; antioxidant activity; topical formulations; sun protection factor (SPF).

# Resumo

*Cynara Cardunculus* var. *Scolymus*, usualmente conhecida por alcachofra, é uma espécie pertencente à família *Asteraceae* e é característica da região Mediterrânea, sendo um dos vegetais amplamente consumidos nessa região. Assim, esta espécie representa uma importante fonte de rendimentos para a agricultura e economia dos países mediterrâneos, sendo Espanha, França, Itália e Grécia responsáveis por mais de 45% da produção mundial de alcachofra.

A alcachofra é maioritariamente composta por água, proteínas, lípidos, hidratos de carbono, inclusive inulina, fibras e açucares, mas também apresenta na sua constituição minerais e vitaminas. Esta espécie é ainda altamente rica em polifenóis, especialmente em derivados de ácido cafeoilquínico, como é o caso do ácido clorogénico (3-*O*-ácido cafeoilquínico) e da cinarina (1,3-ácido cafeoilquínico), e ainda em flavonoides como o cinarósido (7-*O*-glucósido de luteolina). Estes são responsáveis pela atividade antioxidante associada à alcachofra, sendo que quanto maior o seu conteúdo em polifenóis, maior a sua capacidade antioxidante. Para além da sua atividade antioxidante, a alcachofra é conhecida pelas suas amplas propriedades terapêuticas: colerética, hepatoprotetora, anticarcinogénica, antibacteriana, antifúngica, anti-inflamatória e anti envelhecimento.

Os compostos bioativos presentes em tecidos vegetais, tais como os polifenóis, são extraídos através de inúmeras técnicas, sendo que este processo depende de diversos fatores, como é o caso do pH, solubilidade, tempo de extração e temperatura. A escolha do método de extração é fundamental e de extrema importância uma vez que influencia a taxa de extração, o rendimento e a pureza dos compostos extraídos. A infusão é um método de extração convencional, no qual as folhas da espécie em causa são introduzidas em água a ferver por um curto período de tempo, sendo o tempo de extração e a temperatura os parâmetros com maior impacto. Estes métodos de extração resultam em frações com elevado nível de impureza. Assim, a purificação dos extratos através da remoção de materiais maioritariamente inertes é uma opção que visa a obtenção de frações com propriedades melhoradas, incluindo a atividade antioxidante. A digestão ácida com suco gástrico seguida de uma diálise e a precipitação de mucilagens presentes nos extratos vegetais são duas estratégias de purificação.

A aplicação destes compostos em produtos cosméticos tem vindo a aumentar devido à proibição de utilização de ingredientes de origem animal e à procura de produtos naturais e sustentáveis.

As formulações tópicas que têm por base extratos de origem vegetal têm como principais funções a proteção da pele contra efeitos exógenos e endógenos prejudiciais, e a manutenção do equilíbrio hidrolipídico da pele. E estas podem ser líquidas, tais como suspensões, soluções ou emulsões, ou semissólidas, como é o caso de pomadas, cremes e geles. As formulações semissólidas são preferíveis, dado que os ingredientes bioativos quando incorporados nestas podem ter ação nas camadas mais superficiais ou nas camadas mais profundas da pele, dependendo das características físico-químicas dos ingredientes bioativos e da finalidade das formulações, e ainda devido ao seu comportamento reológico. A qualidade, segurança e eficácia destes produtos são avaliadas através de diversos parâmetros físico-químicos, como o pH, homogeneidade e textura, através controlo microbiológico e estudos de irritabilidade cutânea, de citotoxicidade e de estabilidade, e ainda por parâmetros biométricos, tais como perda transepidérmica de água, hidratação, reforço da barreira cutânea, elasticidade, entre outros.

O objetivo deste trabalho foi estudar as propriedades antioxidantes do extrato de alcachofra e a sua utilização como ingrediente para produtos cosméticos antioxidantes.

O presente estudo utilizou folhas de alcachofra, as quais foram submetidas a um processo de infusão, de filtração e liofilização com o intuito de extrair e estabilizar o extrato de alcachofra. A caracterização do extrato de alcachofra foi realizada através de métodos de quantificação específicos e revelou que este é constituído por 62.8% de fibras dietéticas solúveis e insolúveis, 3 % de polifenóis, 0.82% de hidratos de carbono e 0.002% de proteínas.

Duas estratégias de purificação foram adotadas visando o melhoramento das atividades antioxidantes do extrato de alcachofra: digestão ácida seguida de uma diálise com duração de 24 horas que resultou na fração A, e precipitação de mucilagens, na qual se testou ainda a importância da utilização de ácido acético, que resultaram nas frações B (utilização de ácido acético) e C (ausência de ácido acético). Os extratos aquosos procedentes das purificações, bem como o extrato aquoso de alcachofra, foram analisados

através de cromatografia líquida de alta eficiência (HPLC-DAD) com o intuito de acompanhar os processos de purificação e de dosear a cinarina, o ácido clorogénico e o cinarósido presente em cada fração. Estes compostos foram identificados em todas as frações estudadas, sendo que o extrato de alcachofra demonstrou ser o mais rico.

A atividade antioxidante de todas as frações foi avaliada através do ensaio com o radical livre 1,1-diphenyl-2-picrylhydrazyl (DPPH). Todas as frações apresentaram atividade antioxidante, sendo que a fração que apresentou o melhor resultado foi a fração C, resultante da precipitação de mucilagens sem a utilização de ácido acético, com um $EC_{50}$ de 56 μg/mL.

O extrato de alcachofra e a fração C foram estudados de forma mais detalhada. A sua atividade antioxidante foi ainda avaliada através de ensaios *in vitro* em queratinócitos, nos quais foram utilizados como agentes oxidativos o peróxido de hidrogénio e radiação UV. Ambas as frações não apresentaram diferenças significativas relativamente ao ácido ascórbico no caso da radiação UV, mas apresentaram ligeiras diferenças no caso do peróxido de hidrogénio.

O fator de proteção solar (FPS) foi determinado, tendo-se obtido um FPS de 10.99 para o extrato de alcachofra e 10.20 para a fração C. A citotoxicidade foi também avaliada e apesar de se ter observado redução da viabilidade celular, os extratos foram considerados seguros.

Ambas as frações foram incorporadas em dois tipos de formulações tópicas: emulsões O/W e geles. Ambas as formulações foram desenvolvidas com a máxima simplicidade possível, tendo-se utilizado apenas os ingredientes essenciais de forma a diminuir possíveis efeitos secundários provocados por algum ingrediente presente. Para cada formulação foi realizada uma caracterização físico-química (pH, viscosidade), e um controlo microbiológico. O pH ligeiramente acídico, entre 5.4 e 6.4, as propriedades reológicas e a quase total ausência de fungos e bactérias nos produtos cosméticos desenvolvidos, revelaram que estes são adequados e seguros para o uso tópico.

Nos ensaios de citotoxicidade das formulações em queratinócitos observou-se uma diminuição da viabilidade celular associada às formulações que continham os ingredientes bioativos relativamente aos seus respetivos brancos. A nível da integridade membranar, apenas os cremes revelaram diferenças significativas quando comparados

com o seu branco. Assim, as formulações foram consideradas biocompatíveis e seguras para o uso tópico.

Tendo em conta os resultados dos estudos *in vitro* e os custos e tempo de produção de cada fração (extrato de alcachofra e fração C), foi decidido testar apenas as formulações contendo o extrato de alcachofra nos seguintes estudos *in vitro* e nos estudos *in vivo*.

As atividades antioxidantes das formulações com o extrato de alcachofra incorporado foram avaliadas através de ensaios *in vitro* em queratinócitos, nos quais se utilizou peróxido de hidrogénio e radiação UVB como indutores de stress oxidativo. Os resultados demonstraram que ambas as formulações, creme e gel, apresentam atividades antioxidantes elevadas. Nos estudos *in vivo*, foram realizados um *Human Repeat Insult Patch Testing* (HRIPT) e um ensaio para determinar o efeito de proteção das formulações contra stress oxidativo induzido por radiação UV. Os resultados demonstraram que ambas as formulações, o creme e o gel, apresentam uma boa compatibilidade cutânea, e que apesar de ambas as formulações possuírem a capacidade de diminuir a atividade oxidante provocada pela radiação UVA, apenas o gel de alcachofra demonstrou uma atividade antioxidante verdadeira.

O processo de infusão demonstrou ser um método eficaz na extração de polifenóis da alcachofra. O mesmo ocorreu para os processos de purificação realizados, uma vez que estes conseguiram melhorar as atividades antioxidantes de cada fração, mesmo daquelas com um menor conteúdo de polifenóis. Através de ensaios *in vitro*, as formulações desenvolvidas com os extratos eleitos revelaram ser adequadas e seguras para o uso tópico. Posteriormente, estudos *in vivo* também demonstraram que estas formulações eram dermatologicamente seguras, uma vez que não apresentaram reações adversas na pele nem potencial efeito alergénico. Para além disso, estes estudos comprovaram de novo o potencial antioxidante da alcachofra, sendo que o gel que continha o extrato de alcachofra apresentou atividade antioxidante *in vivo*.

Este estudo demonstrou assim o potencial da alcachofra para ser introduzido em produtos cosméticos com finalidades antioxidantes e anti envelhecimento.

Palavras-Chave: *Cynara Cardunculus* var. *Scolymus*; alcachofra; estratégias de purificação; atividade antioxidante; formulações tópicas; fator de proteção solar (FPS).

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# List of Abbreviations

AC – Artichoke Extract Cream

AG – Artichoke Extract Gel

ANOVA – Analysis of variance

BC – Blank Cream

BG – Blank Gel

CC – Artichoke Fraction C Cream

CG – Artichoke Fraction C Gel

DMSO - Dimethyl sulfoxide

DPPH - 1,1-diphenyl-2-picrylhydrazyl

$EC_{50}$ - Half maximal effective concentration

HPLC-DAD – High-performance liquid chromatography–diode array detector

MTT - 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

O/W – oil-in-water

PBS - Phosphate buffer saline

PI – Propidium Iodide

ROS - Reactive oxygen species

SDS - Sodium Dodecyl Sulfate

SPF – Sun Protector Factor

TAMC - Total Aerobic Microbial Count

TYMC - Total Combined Yeasts and Molds Count

UV – Ultraviolet

# 1. Chapter: Introduction

## 1.1. *Cynara Cardunculus* var. *Scolymus*

### 1.1.1. The Domestication of Artichoke

The artichoke was already known and cultivated at the beginning of the Christian era [1]. The first mention to this specie was made by Theophrastus, the Greek (371-287 BCE), who described artichoke cultivation in Sicily and Southern Italy [2], and in 77 EC the medical benefits of this plant were reported by the Roman Pliny the Elder (ad 23–79, in Naturalis Historia). However, it's estimated that domestication of artichoke only occurred between 800 and 1 500 CE, possibly in monastery gardens [3]. Records point Filippo Strozzi as the first merchant of this specie, who was responsible for trading artichokes from Sicily to Florence in the beginning of 15th century [4].

### 1.1.2. Taxonomy and Botanical Description

*Cynara cardunculus* var. *scolymus*, usually known as globe artichoke, belongs to the family *Asteraceae*, subfamily *Carduoideae* and genus *Cynara*. Wiklund used a cladistics method based on morphological characterization to search the ancestry of *Cynara* crops and the results showed that the cultivated artichoke (*C. Cardunculus* var. *Scolymus*), leafy cardoon (*C. cardunculus* var. *Altilis*) and wild cardoon (*C. Cardunculus* var. *Sylvestris*) are included in a single species: *C. Cardunculus L.* [5]. A more recent study analyzed the phyletic relationships between *Cynara* species by using internal transcribed spacer sequences of the ribosomal regions, and the results were nearly in accordance with the phylogeny proposed earlier by Wiklund [6]. In Figure 1.1 are represented harvested artichokes and its habitat.

Figure 1.1 - Harvest globe artichokes at left and plant habit of globe artichoke at right. Illustrations removed from Edible Medicinal And Non-Medicinal Plants.

The globe artichoke is a perennial herbaceous plant, up to 80-180 cm tall, characterized by greyish green and deeply lobed leaves up to 75 cm in length. The flower heads are 5-10 cm in diameter and consists of green bracts surrounding violet florets. Both bracts and florets are attached to a receptacle, commonly known as the heart [7].

At first the leaves of the globe artichoke are rosulate, but later they sprout and adopt an ovate-lanceolate shape. These leaves are silvery-grey, pinnatifid and normally large, up to 50 cm long by 20 wide. This specie is characterized by a voluminous ovate capitula, usually known as the head, which is characterized by a violet-blue flower that is placed on the top of the flowering stem, up to 1.5 m. This head is composed of thick, fleshy bracts called phyllaries (involucral bracts), which are broadly ovate and green or tinged with purple or wholly purple, and a fleshy base of the receptacle, also called heart. These are the edible parts of globe artichoke.

### 1.1.3. Agroecology

Globe artichoke has a wide adaptive range of 7-30°C, being a cool-season crop. Therefore, it should be planted in areas with day and night temperatures between 20-22°C and 12-14°C, respectively. This specie is very sensible to lower temperatures, and severe of frequent frosts can damage or kill it; but it tolerates well high temperatures (>30°C), though it tends to decrease the quality of artichoke heads. It also requires long periods of sunlight, having a critical photoperiod of 10.5 hours [8].

The transition from vegetative to reproductive phase in seed-planted individuals depends on the interaction between the size of the plant, low temperatures and photoperiod. This type of artichoke has deep-rooted and can grow in several varieties of soils. However, it grows best on deep, fertile and well-drained soils with a pH range of 6-8. It requires a raised-bed culture when drainage of the field isn’t known, since globe artichoke dislikes water-logged soils [8].

### 1.1.4. Geographic Distribution

Mediterranean diet is characterized by a high consumption of vegetables, fruits, cereals and olive oil, which provide numerous micronutrients, such as vitamins, minerals, fibers

and polyphenols [9]. The artichoke is one of those vegetables, and it is consumed raw, boiled, steamed or fried, and therefore it's used in numerous recipes [10].

This plant plays an important role in the agriculture and economy of the Mediterranean region, with a production of 199 900 t in Spain, 36 423 t in France, 547 799 t in Italy and 28 600 t in Greece; it is also produced on a large scale in the North Africa – 777 671 t. Global production is approximately 1 793 016 t, making Mediterranean countries belonging to Europe – France, Spain, Italy and Greece – responsible for over 45% of artichoke production, which makes it a source of income that is helping the economy in this region [11].

### 1.1.5. Phytochemistry

The head of globe artichoke corresponds to 30-40% of its fresh weight [12] and the receptacle accounts for 33-55% of the head [13]. A raw globe artichoke is manly composed of water (84.94%); but it also contains proteins (3.27%), ash (1.13%), fat (0.15%), carbohydrates (10.51%), including inulin (1.8%), fibers (5.4%) and sugars (0.99%), such as glucose, fructose and sucrose. Minerals (0.66%) and vitamins (0.049%) are as well important components [14], [15].

Carbohydrates are polyhydroxy aldehydes or ketones or substances that can originate such compounds through hydrolysis reactions, and they may be divided into three major classes: monosaccharides, oligosaccharides and polysaccharides. Plant polysaccharides can be classified into storage polysaccharide (e.g. inulin; the body can digest), cell-wall polysaccharides (e.g. cellulose; the body can't digest it) and gums and mucilage. Structural fibers make up the plant cell walls and include lignin, cellulose, hemicelluloses and pectins.

Globe artichoke has high content of carbohydrates, including the storage polysaccharide inulin. Inulin is a fructan-type polysaccharide that consists of (2→1) linked β-d-fructosyl residues (n = 2–60), usually with an (1↔2) α-d-glucose end group (degree of polymerization ranging from 2 to 60 or more). Previous studies report a content between 1.8 and 2.5% of inulin in this plant [16], [17]. Fibers are also one of the most abundant constituents in the artichoke, namely cellulose and lignin. Cellulose is an unbranched glucose water-insoluble polymer with around 3 000 glucose units and it is the most widely

distributed component of plant cell walls, and lignin is phenylpropane polymers synthetized from coumatyl, coniferyl and sinapyl alcohols. Artichoke fibers are composed of 75.3 % of cellulose and 4.3% of lignin, being a rich-fiber plant [18]. Chemical structure of some compounds present in artichoke are showed in Figure 1.2.

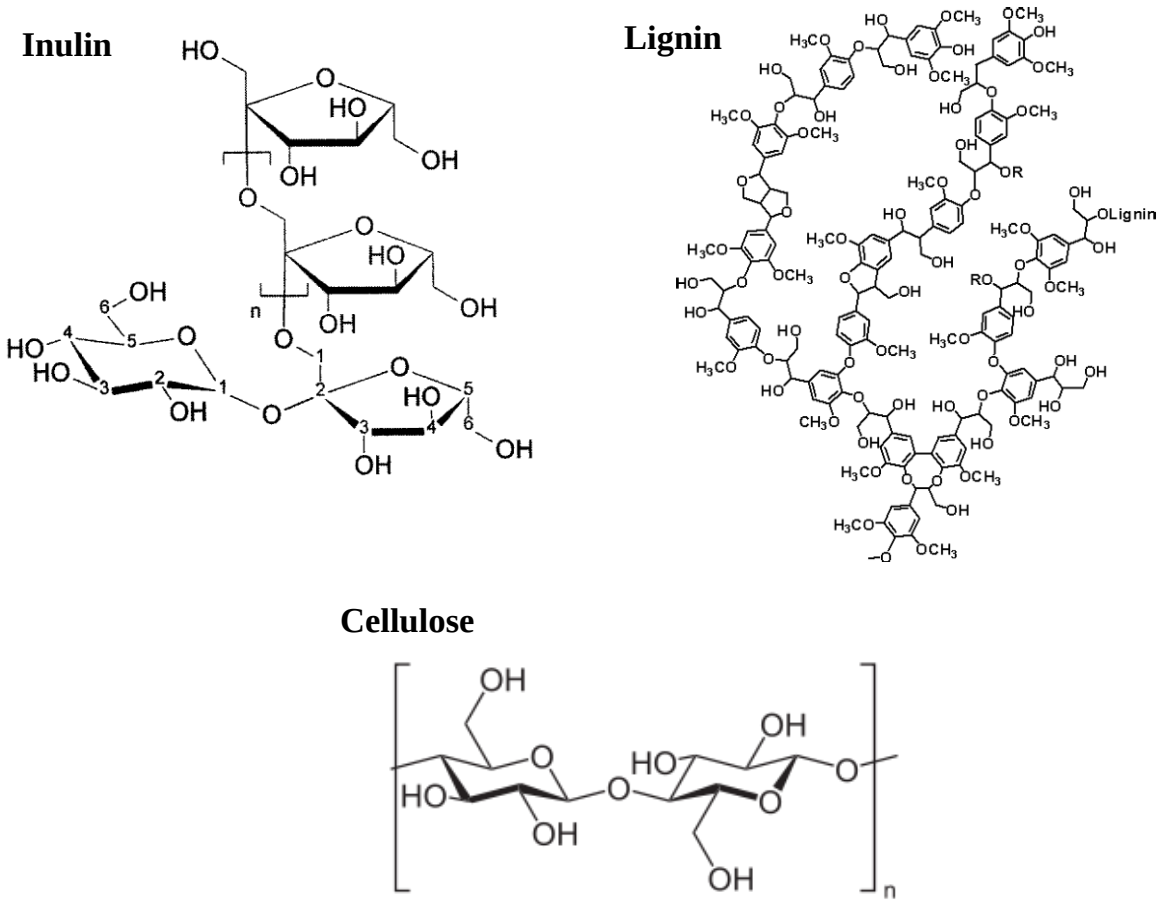


Figure 1.2 - Representative compounds present in the artichoke: inulin, lignin and cellulose.

Phenolic compounds are one of the biggest and widely distributed groups of secondary metabolites in plants. All polyphenols share a common origin, the amino acid phenylalanine, which is deaminated to cinnamic or converted to tyrosine, and then it enters the phenylpropanoid pathway. Therefore, phenolic compounds have as a common characteristic, the presence of at least one aromatic ring hydroxyl-substituted. The polyphenols can be classified into simple phenols, hydroxybenzoic acid and hydroxycinnamic acid derivatives, flavonoids, stilbenes, lignans and hydrolyzable as well as condensed tannins. These compounds are usually bounded to other molecules, commonly sugars and proteins, but they also exist in a small quantity in their free form, possibly due to its toxicity when in free state. They play an important role in plants,

namely in sensorial properties (color, aroma, taste and astringency), structure, pollination, resistance to pests and defense from predators and in grow, germinative and reproduction processes. According to literature, the outer and inner bracts, the receptacle and the leaves of globe artichoke contain polyphenols [10], [19], but its content can be influenced by the harvest time. The phenolic content can increase up to 16 times from winter to Spring harvest, especially in floral stem [20].

Zhu *et al*. [21] identified four caffeoylquinic acid derivatives, chlorogenic acid, cynarin, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid, and four flavonoids, luteolin-7-rutinoside, cynaroside, apigenin-7-rutinoside and apigenin-7-*O*-β-D-glucopyranoside in artichoke leaf extracts, and Falé *et al*. [22] also identified cynaroside (luteolin-7-*O*-glucoside) and luteolin-7-*O*-(6’’malonylglucoside). However, the artichoke extract is mainly composed of caffeoylquinic and dicaffeoylquinic acids, being the most abundant cynarin (1,3-dicaffeoylquinic acid) and chlorogenic acid (3-*O*-caffeoylquinic acid)) [19], [23]. Chemical structure of some phenolic compounds present in artichoke are showed in Figure 1.3.

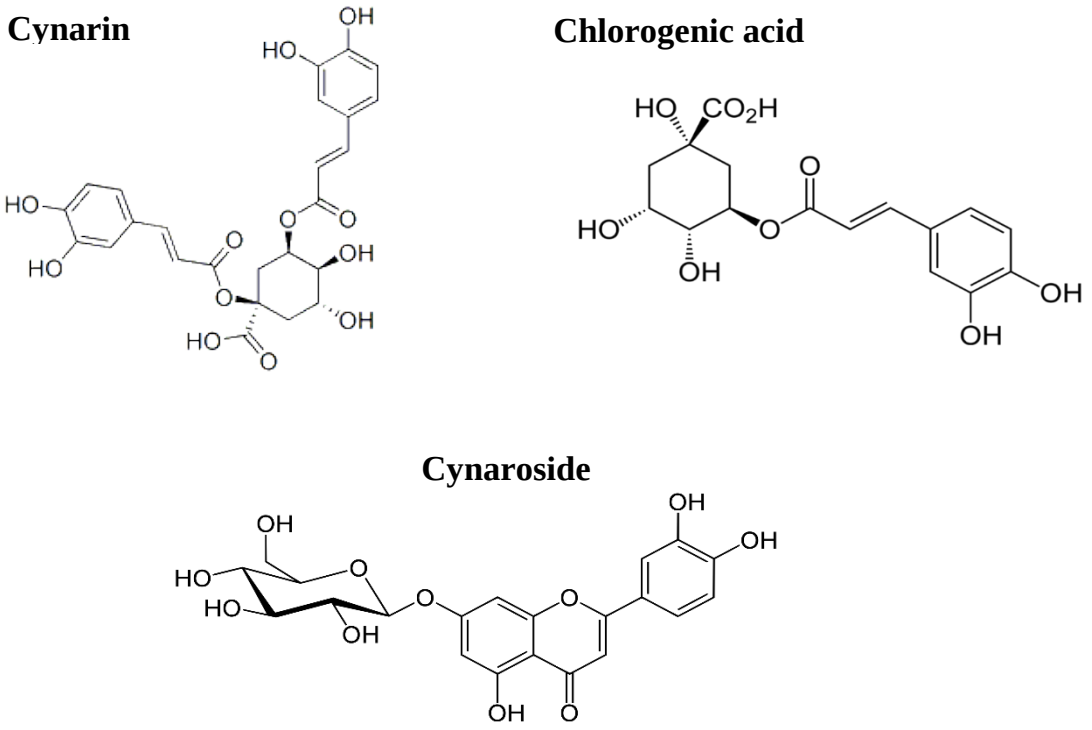


Figure 1.3 - Representative phenolic compounds in artichoke: cynarin, cynaroside and chlorogenic acid.

### 1.1.6. Therapeutic Properties

Artichoke dried leaves have been used as a medicinal herb in traditional medicine for centuries because of its choleretic and hepatoprotective [24], anticarcinogenic[25], antioxidant [19], [22], [26], antibacterial [21], antifungal [27], anti-inflammatory [28] and antiphotoaging properties [29].

The hepatoprotective activity of artichoke was tested in isolated rat hepatocytes against $CCl_4$ toxicity and the results revealed that cynarin and caffeic acid act in glutamine oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GTP), consequently showing hepatoprotective properties, while isochlorogenic acid and cynaroside act only in the GOT activity. Quinic acid and chlorogenic acid didn't alter the toxicity of $CCl_4$ [30]. Another study showed that the artichoke leaf extract with the highest content in phenolic compounds exerted a more significant effect on bile flow and liver protection, but the results also showed that the administration of cynarin and chlorogenic acid as pure compounds didn't revealed hepatoprotective or choleretic properties [24]. The investigators suggested that caffeoyl derivates have an important role in therapeutic properties of artichoke, but when administrated in their pure form, they aren't effective.

Mileo *et al*. [31] investigated the chemopreventive properties of artichoke edible parts through cytotoxicity assays on several cell lines and the results showed a similar level of toxicity in breast cancer cell lines but not any notable effects, even at high concentrations, on normal breast epithelial cells. This effect is due to the combined activities of several bioactive compounds present in the edible part of artichoke, since a study demonstrated that the artichoke extract has a faster and more pronounced apoptotic activity than the pure chlorogenic acid [32].

Through the measurement of the lag phase prolongation of conjugated diene formation, the decrease in the rate of propagation and a sparing effect on the a-tocopherol within the low density lipoprotein (LDL), artichoke extract showed to have the capacity to retard LDL oxidation in a dose-depend way. Pure luteolin revealed a lipid peroxidation inhibition similar to 20 μg/mL of artichoke extract, and luteolin-7-*O*-glucosine also showed a dose-dependent inhibition of LDL oxidation but was less effective than luteolin. Studies of cooper-chelating properties of these compounds suggested a potential role in the antioxidant activity of artichoke extract [33]. Artichoke has an $EC_{50}$ of 123.1 ± 5.7 μg/mL for DPPH scavenging activity and its biochemical activities (acetylcholinesterase

inhibition and DPPH scavenging activity) remain similar after gastric and pancreatic digestions [22].

In order to examine the antimicrobial activities of artichoke leaf extract, three fractions of it in different solvents, chloroform, ethyl acetate and n-butanol, were tested by disk assays. The results demonstrated that at least six types of bacteria, including *B. subtilis, S. aureus, A. tumefaciens* and *M. luteus,* and five fungi, *including C. albicans, S. cereVisiae, S. carlsbergensis, A. niger* and *P. oxalicum,* were susceptible to all artichoke fractions [21]. However, *C. lusitaniae* and *M. mucedo* were only susceptible to n-butanol fraction and *C. cucumerinum* was sensitive only to n-butanol and chloroform fractions. Therefore, the n-butanol fraction was considered the most active to all microorganisms tested. In the same study, eight phenolic compounds were isolated from n-butanol fraction, including chlorogenic acid, cynarin, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, luteolin-7-rutinoside, cynaroside, apigenin-7-rutinoside and apigenin-7-*O*-β-D-glucopyranoside, and the compounds that showed a higher antimicrobial activity were the chlorogenic acid, the cynarin, cynaroside and luteolin-7-rutinoside. In following studies, artichoke leaf extract also showed antifungal activity against 7 foodborne bacterial pathogens, *Bacillus subtilis, Staphylococcus aureus, Agrobacterium tumefaciens, Micrococcus luteus, Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa,* 4 yeasts, *Candida albicans, Candida lusitaniae , Saccharomyces cerevisiae* and *Saccharo myces carlsbergensis* , and 4 molds, *Aspergillus niger , Penicillium oxalicum , Mucor mucedo and Cladosporium cucumerinum* [27].

A group of 10 albino mice were administrated via oral with 10% infusion (dry plant) or 20% (fresh plant) corresponding to 1 or 2 g/kg of several plants, and the analgesic and anti-inflammatory activities were measured by the number of contortions and Evans blue dye diffusion to the peritoneal cavity, respectively [34]. From 10 plants tested, artichoke was one of the two plants that showed powerful anti-inflammatory and analgesic activities [28]. In other study, elevated levels of proinflammatory cytokines in blood serum of patients with chronic viral hepatitis C were normalized after using artichoke preparations during the medical rehabilitation period.

Cynaropicrin is a bioactive sesquiterpene lactone and its content in artichoke extracts ranges between 0.44-1.60 % [35]. The administration of this compound found to be

effective against photoaging of the skin through the inhibition of the nuclear factor kappa (NF-κB)-mediated transactivation of basic fibroblast growth factor (bFGF) and the matrix metalloprotease-1 (MMP-1) in a dose-dependent manner [29]. NF- κB is normally retained in the cytoplasm as an inactive complex but it is activated by UV radiation, which induces bFGF and MMP-1 production [36], [37]. Was also confirmed that in an *in vivo* mouse model, cynaropicrin is able to prevent skin photoaging, which is traduced in a hyperproliferation of melanocytes and keratinocytes [29].

### 1.1.7. Artichoke Waste Phytochemicals

The waste produced by the artichoke processing industry, such as leaves, outer bracts and stems, can form up to 80-85% of total biomass of the material, and it is normally used as organic mass, animal feed [38], and fuel [39] and fiber production [40].

In the artichoke waste were reported 45 phenolic compounds, including gallic acid; caffeoylquinic acid 1,2 and 3; dicaffeoylquinic acids 1,2,3,4,5,6 and 7; protocatechuic acid; esculin; chlorogenic acid; p-coumaric acid-*O*-glucoside isomers 1 and 2; eriodictyolglucuronide; rutin (quercetin-3-*O*-rutinoside); dicaffeoylquinic acid derivative; hyperoside (quercetin-3-*O*-galactoside); luteolin-7-*O*-rutinoside; cynaroside (luteolin-7-*O*-glucoside); isoquercitrin (quercetin-3-*O*-glucoside); luteolin-7-*O*-glucuronide; luteolin-7-*O*-galactoside; naringenin-*O*-hexoside; avicularin (quercetin-3-*O*-arabinoside); isorhoifolin (apigenin-7-*O*-rutinoside); quercetin-*O*-pentoside; quercitrin (quercetin-3-*O*-rhamnoside); luteolin-7-*O*-neohesperidoside; apigenin-*O*-glucoside; prunin (naringenin-7-*O*-glucoside); naringin (naringenin-7-*O*-neohesperidoside); scolimoside (luteolin-7-*O*-rhamnoside); apigenin-7-*O*-glucuronide; quercetin-*O*-pentoside; phloridzin (phloretin-2-*O*-glucoside); feruloylquinic acid-*O*-glucoside; luteolin; quercetin; naringenin; and apigenin chrysoeriol. Among all components, caffeoylquinic and dicaffeoylquinic acids showed to be the main components of artichoke waste [41].

Knowing the therapeutic properties of all artichoke components combined, the actual use of artichoke by-products is questionable and since it has several health benefits, alternatives for its use should be considered. Production of natural nontoxic food additives, neutraceuticals or cosmetics using artichoke as bioactive ingredient are some options for the use or artichoke by-products.

## 1.2. Purification Methods

Extraction is the separation of active ingredients of plant tissues from the inactive or inert compounds through the use of selective solvents in standard extraction techniques. The plant extracts obtained are still a relatively impure liquid, semisolid or powders and include several chemical compounds with different functions and structures, such as polyphenols [42]. The extraction of polyphenols from leaves depends on many factors including pH, solubility, extraction time and temperature [43]. However, the extraction method has effects on the rate, yield and purity of these compounds, so the method choice is crucial.

Infusion is a conventional method, in which dried leaves are placed in boiling water for a short period of time. The temperature and extraction time are parameters with impact in the total polyphenol content and consequently in antioxidant activity [42]. Liang *et al*. [44] investigated the influence of temperature on the extraction efficiency and degradation of polyphenols in green tea. The results showed that at a temperature of 100°C, the green tea catechins suffered alterations on their configuration to their isomers, and at 80°C the catechins epimerization was inhibited. Therefore, the extraction should be executed at a temperature between 80 and 100°C. Another study showed that a prolonged extraction period leads to the degradation of a large fraction of flavonoids present in green tea [45]. The investigators concluded that the conventional procedure of pouring boiling water to the cup with the herbal leaves and let it rest for 10 minutes is the proper way to infuse the herbs. This extraction technique is usually followed by a filtration procedure in order to separate the plant tissues from the solution containing the bioactive compounds. The vacuum filtration is a well-know and simple method that consists in passing the stream through a porous filter, which is capable of retaining the solids. This filtration requires a pressure drop in order for the liquid to flow through the filter, and this pressure can be achieved by creating a vacuum or a centrifugal force, by raising the pressure more than the atmospheric pressure or by designing to make use of gravitational force [46]. The vacuum filtration schematic is represented in Figure 1.4.

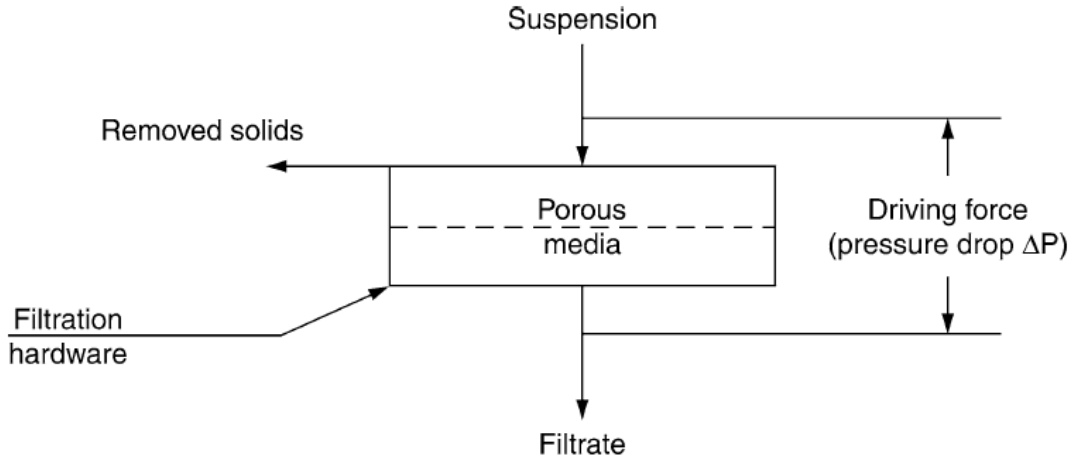


Figure 1.4- Flow diagram of a vacuum filtration system. Illustration removed from Biosolids Tratment Processes.

To finalize the active substances extraction procedure from herbs, a lyophilization of the herb infusion is required to remove its water content, which enhances its stability. To proceed to the lyophilization, the herbal infusion needs to be frozen in order to separate the solvent from the solutes, since the water forms ice crystals and the solutes will be confined in the interstitial region between those crystals. Then the pressure in the system is reduced and heat is applied to the frozen herbal infusion, which will lead to the sublimation of the ice crystals formed previously. A dried mixture, usually named cake, is the result of this process, but it may contain 5-10% (w/w) of water adsorbed onto its surface. In some cases, if the water content in the cake is still too high and consequently its stability isn't the desired, a second drying is performed through the increasing of temperature and reducing of partial pressure of water vapor in the container [47]. The diagram of a lyophilization is represented in Figure 1.5.

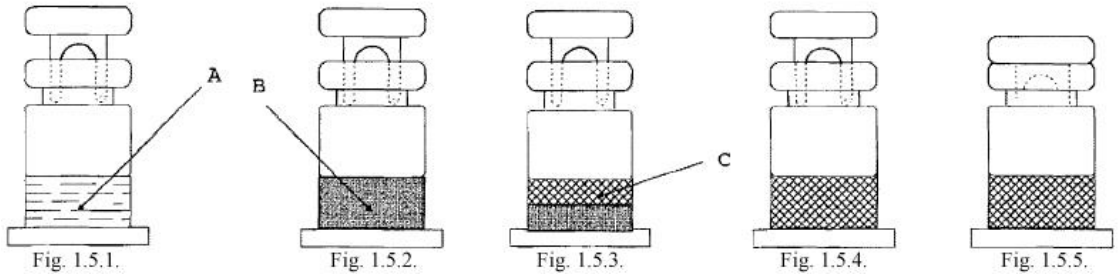


Figure 1.5 - Diagram of lyophilization steps of a liquid formulation. Figure 1.5.1. shows the liquid formulation, which is market by 'A', in a glass container with the lyophilization closure positioned for the drying process; Figure 1.5.2. shows the frozen ice-product matrix of the formulation, which is market by 'B'; Figure 1.5.3. represents the primary drying process and the interstitial cake portion is denoted by region market as 'C'; Figure 1.5.4. illustrated the completion of secondary drying. Figure 1.5.5. demonstrates the final product with the closure in its stoppered position. Illustration adapted from Lyophilization: introduction and basic principles.

The herbal extracts obtained from the previous method are still impure, so a purification is required in order to enhance its properties, including the antioxidant activity. Two different approaches can be followed in order to purify the herb infusion: gastric digestion followed by dialysis and mucilage precipitation.

Tagliazuchi *et al*. [48] investigated the bio-accessibility of the major classes of polyphenols from Red Globe grapes through an *in vitro* model that simulated some chemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) gastro-intestinal conditions. The investigators concluded that the content of bio-accessible polyphenols, flavonoids and anthocyanins increases during gastric digestion, and that the gastric digestion has no effect on the stability of polyphenols. Therefore, it is advantageous to perform a gastric digestion of infusions in order to increase its bio-accessible polyphenols content, which will consequently improve its health benefits. Afterwards, a dialysis is required to separate the polyphenols from other large and complex molecules present in the herbal infusion, and consequently purify it. Dialysis is a separation method, based on the differential diffusion, of solutes with different molecular size through a membrane. This procedure is considered slow but it is efficient in separating small solutes from large molecules, and due to its simplicity, it is a method of choice. In this technique, the solution to be dialyzed is inserted in a dialysis membrane and then it is placed in a container with water for some hours or even days. In this process, a way of stirring the solutions is advisable and for that purpose, a magnetic stirred can be used to gently move continuously the dialysis membrane [49].

Another purification method is the precipitation of mucilage, a polysaccharide mixture that can be found in plant tissues of higher plants, present in herbal infusions. Kim *et al*. [50] investigated the effects of pectic mucilage removal from cactus cladodes (*Opuntia humifusa* Raf) and the results showed that along with the mucilage elimination occurs an increase of total polyphenols, and since these compounds contribute to antioxidant activity, it also resulted in an enhanced antioxidant activity.

## 1.3. Oxidative Stress in Skin

The skin covers the entire surface of our bodies and it corresponds approximately to 15% of total adult body weight, being the biggest organ in human body [51]. It consists of differentiated cells and tissues that perform several functions in our body, such as defense against other organisms, maintenance of body temperature and protection from external environment [52]. The protection of external physical, chemical and biological aggressions is due to a complex structure that has different tissues, like epithelial, connective, vascular, muscular and nervous, which are organized in three distinct layers: epidermis, dermis and hypodermis [51]. The skin also has several specialized structures with different functions called appendages (sebaceous, eccrine sweat and apocrine glands, and hair follicles). The epidermis is a tough stratified epithelium composed of cells with different embryonic origins, such as melanocytes, Langerhans cells, Merkel cells and mostly keratinocytes. It exhibits a progressive differentiation (keratinization and cornification) from the basal to the surface, which results in different appearances of cells from one layer to another [52]. Basal cells are attached to an underlying basal lamina in the innermost layer, and above this one is the stratum spinosum composed of prickle cells. Upward is a thin layer consisting of granular cells - stratum granulosum - which form a waterproof barrier, being this one of the most vital functions of the epidermis. This layer is attached to the outermost layer called corneal stratum, which consists of dead cell with no intracellular organelles. This cells rich in keratin are connected by a thin and tough layer of proteins, including cytoplasmic protein involucrin [53]. Epidermis is associated with some appendages like pisosevaceous follicles and sweat glands [51]. Skin layers are represented in Figure 1.6.

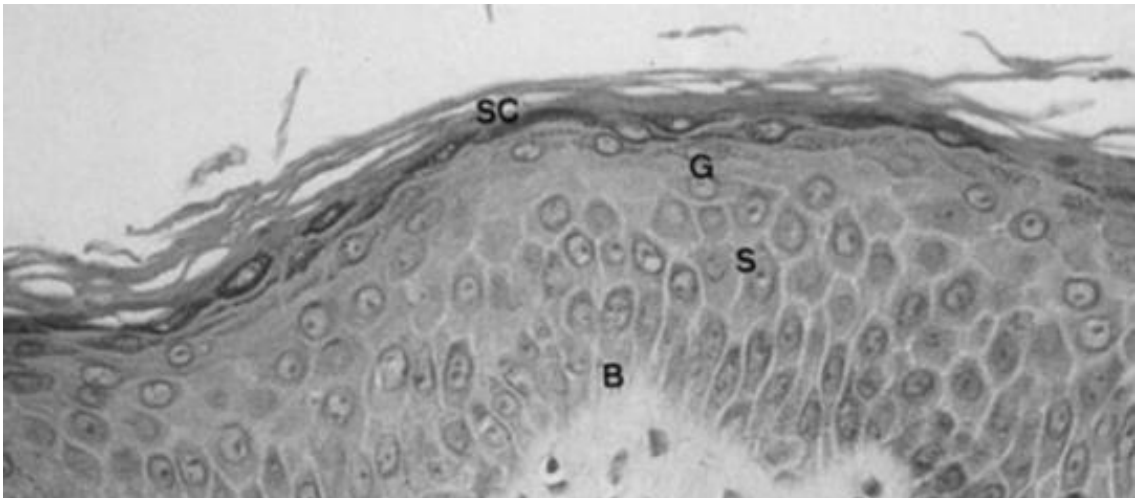


Figure 1.6 - Basal cell layer (B), spinous layer (S), granylar layer (G) and stratum corneum in a hemtoxylin and eosin stains section of normal skin. Illustration from Freinkel et al, 2001.

Below the epidermis is the dermis, a connective tissue component of the skin that protects the epidermis and provides elasticity and support [51]. The dermis has less cells than the epidermis and it is composed of fibrous molecules, dermal cells, a ground substance, appendances, neurovascular networks and sensory receptors. Its function is to protect the body from physical injuries, to retain water, regulate the temperature and it has receptors of sensory regulation [52]. It has a continuous turnover system that regulates the synthesis and degradation of its protein and it's composed of the papillary and reticular layers [54]. Papillary layer is the upper layer and it is formed of thin collagen fibrils that form thicker collagen fibers [54], cells (fibroblasts, dermal dendrocytes, mast cells), vessels and nerve endings, being this layer the connection between the dermis and epidermis [51]. Bellow this one is the reticular layer that is composed mostly of type I collagen and fibers, and has only about 15% of dermal collagen which is mostly found in the papillary layer [54]. The deepest part of the appendages, vascular and nerve plexures are retain in this last layer [51]. The deepest layer of the skin is the hypodermis that serves as a reserve of energy, protects the skin from mechanical injuries and has a role in thermoregulation [52]. This layer is mostly composed of large adipocytes with a very weakly osmiophilic cytoplasm, which show pericellular expression of S100 protein and vimentin [51]. The adipocytes are organized in primary and secondary lobules separated by connective tissue septa containing cells, such as fibroblasts, dendrocytes and mast cells [51]. The fat cells are in continuous renovation through the accumulation of lipids on their inside, proliferation of adipocytes and recruitment of undifferentiated cells from mesoderm. This continuous system appears to be regulated by a hormone released from adipocytes called leptin [52]. The hypodermis also accommodates vessels, nerves and the deepest part of sweat glands [51].

In 1955, Denham Harman articulated an innovated biochemistry theory that suggested a correlation between oxygen radicals produced by the cells and the aging process. According to his theory, oxygen radicals such as $HO\cdot$ and $HO_2\cdot$ were generated inside cells, most likely due to the interaction of respiratory enzymes and the molecular oxygen and to the action of catalase on hydrogen peroxide, and they were responsible for damages in DNA and proteins, and consequently for the ageing and cancer [55]. Despite the controversy around this theory back in those days, in the present day it is well proved. Skin is exposed to several harmful agents, such as ultraviolet (UV) and ionizing radiation, air pollution and biologic contaminants, that contribute for the production of reactive

oxygen species (ROS) in epidermal tissues. ROS are compounds derived from molecular oxygen, $O_2$, but due to chemical reactions they have extra electrons, which make them unstable. Hydroxyl radicals, $HO_{\cdot}$, are a result from homolytic cleavage of hydrogen peroxide, $H_2O_2$, and superoxide, $O_2\cdot$, a result of molecular oxygen reduction. These two radicals and hydrogen peroxide are largely produced by the cells as a result of several biological mechanisms [56].

Among differentiated cells, human keranocytes are the most exposed to harmful agents, including oxidative stress associated with ROS overproduction, and they are further modulated by exposure to UV radiation, chemical compounds, rich-oxygen environment and inflammatory processes. Orciani *et al*. [57] determined the susceptibility of isolated mesenchymal stem cells from human skin (S-MSCs) to oxidative stress and compared the results with keratinocytes, which are differentiated cells of the same lineage. Cells were exposure to $H_2O_2$, a stress oxidative inductor, for 2 hours and the oxidative stress effects were analyzed after 4, 12, 24 and 48 hours of recovery, and the data reported a greater antioxidant defense for the keranocytes, while stem cells, which are surrounded by a protective environment, weren't able to overcome oxidative stress. However, human keranocytes can still suffer damage at a long-term exposition to harmful agents, like UV radiation [58].

At high levels, ROS can induce damage to cell structures, lipids, nucleic acids and proteins, which can contribute to mutagenesis, carcinogenesis and ageing [59]. Human organism have developed several defensive strategies, like preventative and repair mechanisms, and physical and oxidant defenses [59], but these are not always capable to respond to an ROS overproduction and occasionally some errors may occur. Therefore, the search for new compounds with the capacity of preventing these oxidative stress events is constant.

UV radiation is divided in three distinct types of radiation: UVC (wavelength 200-280), UVB (wavelength 290-315nm) and UVA (315-400nm). The UVB and UVC are mostly filtered by the ozone layer, therefore only 5-10% of highly energetic UVB reaches the skin, being the rest UVA radiation [60]. The UVB radiation is mainly absorbed by the epidermis, while UVA radiation is able to penetrate into the dermis and it also interacts with the stratum corneum and epidermis [61]. There are some chromophores in the skin that can absorb UV radiation, such as melanin, DNA, RNA, proteins, lipids, aromatic

amino acids – tyrosine and tryptophan – and urocanic acid [62]. This absorption can lead to several photochemical reactions and secondary interactions with ROS, which can result in inflammation, photoaging, immunosuppression and skin cancers – cutaneous malignant melanoma, basal cell carcinoma and squamous cell carcinoma [61]. UVA and UVB radiation have different wavelength and therefore, they show distinct properties concerning their biological effects on our skin, being UVB radiation more mutagenic and cytotoxic than UVA. UVA radiation interacts with endogenous photosentisizers resulting in ROS production, which may cause damage to DNA, proteins and membranes. On the contrary, UVB radiation is highly absorbed by DNA resulting in its disruption and the production of photoproducts like cyclobutane pyrimidine dimers (CPD) and pyrumidone. A consequence of these photoreactions is the accumulation of defective DNA that can be the trigger to mutations of genes that regulate the tissue homeostasis and genome integrity, for example the p53 tumor suppressor gene [60]. UVB radiation modifies the epidermal morphology, including the thickness increase of the corneal stratum, modification of cell cohesion and mechanical integrity of corneal stratum and disruption of the permeability barrier, resulting in transepidermal water loss, changes in the lipids of corneal stratum and decreased corneal stratum hydration [61].

Polyphenols from plant tissues have been described as powerful antioxidants that can support the skin's own antioxidant defense against oxidative stress when applied topically [63], [64]. These compounds also have photoprotective properties [65] and sun protection factor (SPF), which ranges between 7 and 29 for stilbenes, flavonoids and hydroxycinnamic acid homologues [66].

## 1.4. Topical Formulations

Nowadays, plant extracts are being more used in cosmetic formulation, mostly due to the prohibition of the use of animal origin ingredients and to an increase search for ecofriendly and sustainable products. These plant extracts are very different from purified therapeutic agents, since plant extracts are more dilute and usually contain more bioactive ingredients that may be related chemically and therapeutically to the main component responsible for the desired effect [67]. The use of bioactive ingredients or phytochemicals extracted from plant tissues in cosmetics have two major purposes, including body care and as a source of nutrients for a healthy skin [68].

In cosmetics is possible to use the entire plant extract, which is mostly applied according with its known therapeutic effects, or selective plant extract, which is used according to the investigations studies on its properties [67]. Usually, these botanical products are rich in vitamins, hydrocolloids, proteins, antioxidants, essential oils and other bioactive compounds [69]. Cosmetic formulations containing bioactive ingredients from a natural source are designed to protect skin against exogenous or endogenous harmful agents and to equilibrate the dermal hydrolipid content, which suffers alterations due to ageing and dermatosis [67].

To develop a good formulation, simplicity is required and the shorter the ingredient list, the better, since it diminishes the possible adverse effects due to a compound present in it and the costs of production. The formulation design has to balance the ingredients used in order to create a product stable and easy to apply, and that maintains its functions during manufacturing, on the shelf and during and after application.

Topical dermatological products can be administered easily and they can be liquid, such as suspensions, solutions and emulsions, or semisolid, including ointments, gels and creams. Active ingredients incorporated in topical formulations can stay on the surface layers of tissues or can penetrate into deeper layers, depending on its physicochemical properties, its action and the formulation approach.

Creams are semisolid emulsions made with two immiscible liquids, water and oil, in which one is considered the dispersion phase and it is dispersed into the other phase, which acts as the dispersion medium, resulting in a stable dispersion. The main ingredients of these formulations are emollients, humectants, surfactants, preservatives, chelating agents, perfumes and others. The equilibrium between water and oily ingredients may produce different formulations in order to suit different purposes (skin types, skin conditions, age of user and living environment). Usually, the main functions of creams are to maintain the moisture balance and keep the skin moist and supple by supplying water, humectants and oils [70]. There are two categories of creams according to the surfactants and oily ingredients used: O/W or W/O emulsions. In O/W emulsions are used hydrophilic surfactants and the oily ingredients can vary widely from non-polar to polar. On the contrary, in W/O emulsions are mostly used lipophilic surfactants and the oily ingredients are mainly non-polar. The W/O type has been used to increase the oily nature and the O/W type when a light feeling is wanted [70].

Gels are a type of base with a uniform external appearance that can vary from transparent to semitransparent and they provide a moist feeling. These formulations can be divided into two categories, aqueous or oily gels. Aqueous gels are used as a base material and are known for their water supplying, moisturizing and cooling effects. Oily gels supply oil to the skin and have moisturizing properties as well. The main compounds of gels are polymers and solvents [70]. Polymers are a crucial gel constituent and they can be classified based on source (natural, semi-synthetic and synthetic), structure (linear, branched chain, crosslinked or network polymer), polymerization type (addition or condensation polymers), molecular forces (elastomers, fibers, thermosetting, thermoplastic), chain growth polymerization (free radicals governed) or degradability (biodegradable or non-degradable) [71]. Water soluble polymers can be applied in several industries, like food, paint, textiles, paper, pharmaceuticals, cosmetics, water treatment, among others, and can be divided into two categories, synthetic or natural. Synthetic water soluble polymers can be dissolve, disperse or swell in water and can work as a gelation, thickening or emulsification/stabilization agent, which result in alterations of physical properties of aqueous systems. These include poly(ethylene glycol) (PEG), polyvinyl pyrrolidone (PVP), Polyvinyl alcohol (PVA), Polyacrylamides, Polyacrylic acid (PAA), N-(2-Hydroxypropyl) methacrylamide (HPMA), Divinyl Ether-Maleic Anhydride (DIVEMA), polyoxazoline, Polyphosphates and Polyphosphazenes; and natural water soluble polymers include xanthan gum, pectins, chitosan derivatives, dextran, carrageenan, guar gum, cellulose ethers, hyaluronic acid (HA), albumin and starch or starch based derivatives [71].

The quality, safety and efficacy of a topical formulation is evaluated by physical and chemical parameters such as pH, homogeneity, texture, microbiological control, skin irritation studies, cytotoxicity, stability and biometric parameters, including transepidermal water loss, hydration, strengthening the cutaneous barrier and elasticity, among others.

Under normal physiological condition, skin has an acidic pH between 4.5 and 6.5 that depends on the region. The acidic skin pH contributes to the defense against microbiological or chemical agents, the skin barrier homeostasis and stratum corneum desquamation [72]. Therefore, since skin pH has several important biological functions and its change could compromise the functions of topical formulations, these should present a pH value similar to skin pH.

Rheology, the science that studies the behavior of materials when they are under the influence of external forces, is a fundamental parameter in the development of formulations, but also in its manufacturing and application, and to ensure long shelf-life. Viscosity and elasticity are the simplest rheological properties [73]. Newtonian fluids are viscous but not elastic materials, like water, that under the smallest force, spend all energy in flowing; and Hookian bodies are elastic but not viscous materials, like rubber, that spends the force applied to change its shape and stores the energy. The semi-solid formulations have both viscosity and elasticity, being characterized by complex rheological properties, which depends on their internal structure [70].

Different rheological fluid behaviors are observed in topical formulations: shearthinning, dilatant fluids, thixotropic and rheopectic. A purely viscous fluid is said to be shearthinning and is characterized by a viscosity decrease along with a shear rate increase. In dilatant fluids, the apparent viscosity increases with increasing shear rate. Shearthinning and dilatant fluids are independent of time. At a constant shear rate, the shear stress decreases monotonically in the case of thixotropic fluids and the shear stress increases monotonically in rheopectic fluids. These last types are time dependent fluids [74].

Topical formulations contain several ingredients, like oil, water and glycerin (source of carbon), and substances, like amino acids derivatives and proteins (source of nitrogen), that can encourage the growth of microorganism. Therefore, to assure its quality is necessary to add preservatives, which are able to suppress the proliferation of microorganisms that may have contaminated it and to kill them, thereby preventing the product deterioration. To assure the near or total absence of microorganisms, two methods are largely used in microbiological control: total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC) tests [70].

Human repeated insult patch testes (HRIPT) is one of the most used methods to evaluate the safety of finished cosmetics products, which is performed under exaggerated conditions of product exposure. This test requires a large group of subjects (100) and can be performed in several different manners with distinct exposure times and occluding methods. The subjects are exposed to the cosmetic products during a 3 weeks' period, which is followed by a 2 weeks' rest period. Afterwards, the challenge period is performed using a 24 or 48 h patch test and the resulting reaction is categorized for clinical

signs. The product concentration used is determined by prior sensitization test results performed in animals, results from repeated application irritation patch studies in humans and prior experience [75].

To study the efficacy of cosmetic products are used non-invasive techniques, such as elasticity, skin thickness, wrinkling, roughness, transepidermal water loss, hydration, strengthening the cutaneous barrier and elasticity, among others. These parameters can evaluate possible adverse reactions and changes that may occurred in the skin, hair or nails after the use of a cosmetic product.

A cosmetic to be considered safe according to regulation (EC) N° 1223/2009, shouldn't show adverse effects on normal or sensitive individuals like skin irritation, sensitization, phototoxicity and photosensitization.

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# 2. Objectives

The aim of this study was to investigate the antioxidant properties of artichoke extract in topical formulations.

For this purpose, other tasks with a high interest were also proposed:

- Increase the acknowledge of purification strategies of plant extracts that may be used posteriorly in other species studies and even in industrial processes;
- Found new applications for this specie since it is highly produced and has an enormous economic value in the Mediterranean region.

# 3. Chapter: Materials and Methods

## 3.1. Purification and Chemical Analysis

### 3.1.1. Preparation of Artichoke Extract

*Cynara Cardunculus Scolymus* bracts from DIATÉTICA® were acquired and an infusion with 91.02 g of it in 910.2 mL of boiled distilled water was performed for 10 minutes. This process was followed by a vacuum filtration with a 70 MM filter in order to remove the bracts from the aqueous part.

### 3.1.2. Artichoke Extract Dialysis

A gastric juice solution was prepared through the addition of 320 mg of pepsin and 200 mg of NaCl to 100 mL of distilled water with a pH of 1.2, which was previously acidified with HCl 37%, and was also prepared a 50 mg/mL artichoke solution. A dialysis mix was prepared with 50% of gastric juice and 50% of artichoke solution, and then put in a bath at 37°C for 60 minutes. Afterward, this mix was added to a dialysis membrane, which was place inside of a goblet with 100 mL of acidified distilled water (pH 1.2) at room temperature. The dialysis process lasted 24 hours. The outer waters were exchanged a few times to prevent its saturation and were preserved in a recipient, and aliquots were collected during the process to evaluate its efficacy.

### 3.1.3. Mucilage Precipitation

In order to precipitate the mucilage present in the artichoke extract, two different approaches were adopted: a method found in literature using acetic acid [1] and the same method readapted without the use of acetic acid. 0.5 g of artichoke extract were dissolved in 25 mL of distilled water, and then this solution was added to 100 mL of A solution (96% ethanol and 1% 100% glacial acetic acid) or B solution (96% ethanol), according to the methodology chosen. A centrifugation at 2 600 g for 10 minutes was followed. The supernatant was separated from the pellet and reserved in a recipient for posterior lyophilization. The pellet was dried in a nitrogen line. The initial mass wasn't always the same, but the reagent proportions used were always equal. The assay with use of acetic acid resulted in artichoke fraction B and the assay with no use of acetic acid in artichoke fraction C.

### 3.1.4. McCleary Method

The McCleary method (AOAC 2009.01) was also performed with the purpose of precipitate the mucilage. The artichoke fraction C obtained from the mucilage precipitation method with no use of acetic acid, was the fraction chosen to perform this method.

Initially, 30 mg of artichoke fraction C were dissolved in 2 mL of distilled water with a pH of 1.2 and then 2 mL of gastric juice were added, and then the mix was place in a water bath for 60 minutes at 37°C. After the first digestion, the mix was placed in the refrigerator for 60 minutes. A centrifugation at 2 600 g for 10 minutes at 4°C was followed, but it didn't form any pellet. Afterwards, another digestion was performed adding 2 mL of pancreatic juice to the mix again. The pH of this mixture was adjusted to 6.0 through the addition of 1 M NaOH. The mix was placed again in the water bath at 37°C for 60 minutes, and then reserved again in the refrigerator for 18 hours. A centrifugation with the same conditions as before was realized, and the supernatant was separated from the pellet for posterior lyophilization and the pellet was dried in an azote line.

The gastric juice was prepared adding 320 mg of pure pepsin and 200 mg of NaCl to a total volume of 100 mL of distilled water previously acidified with 37% HCl to a pH of 1.2. The pancreatic juice was prepared adding 250 mg of pancreatin from porcine pancreas (8 × USP specifications) to a 50 M sodium phosphate buffer solution pH 8, which was prepared using 5 mL of $Na_2HPO_4$ sodium phosphate buffer solution and 5 mL of $NaHPO_4$ sodium phosphate buffer solution.

### 3.1.4. Samples Drying

#### 3.1.4.1. Lyophilization

All samples obtained throughout the study were lyophilized in order to prevent its degradation. The samples were put in falcons or plastic recipients and then were lyophilized until its total dryness in a Heto PowerDry LL3000.

#### 3.1.4.2. Solvent evaporation with rotary evaporator

In order to separate the fractions from undesirable compounds, a solvent evaporation with rotary evaporator was performed at 60°C in a Büchi Rotavapor R-200. The period of time varied according with the time needed to evaporate all liquids present in the solution.

### 3.1.5. High-Performance Liquid Chromatography (HPLC-DAD)

HPLC-DAD analysis were performed in order to quantify the chlorogenic acid, cynaroside and cynarin and to observe the compounds present in the dry extracts. The HPLC-DAD analysis was performed in an Elite LaChrom® VWR Hitachi liquid chromatograph equipped with a column oven L-2300 and Diode array detector L-2455 (VWR, USA). The column LiChroCART® 250-4 LiChospher® 100 RP-8 (5 μm) was used.

The method was already described [2]. It was used an injection volume of 25 μL with an auto injector and a flow rate of 1 mL/min. The detection was carried out between 200 and 500 nm with a diode array detector. A gradient composed by solutions A (0.05% trifluoroacetic acid) and B (methanol) was used according as followed: 0 min, 80% A, 20% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B.

To quantify the chlorogenic acid, cynaroside and cynarin in the extracts obtained during the study, calibration curves were made using two different concentrations: 0.5 and 0.1 mg/mL for chlorogenic acid and cynarin; 0.25 and 0.1 mg/mL for cynaroside.

### 3.1.6. Total Phenolic Content with Folin-Ciocalteau Method

The content of phenolic compounds in the fractions studied was determined according to the Folin-Ciocalteu procedure, using pure gallic acid as standard [3].

The calibration standard curve was obtained using six different concentrations of gallic acid (10, 20, 50, 100, 350 and 500 μg/ mL) and for blank was chosen the same solution without any gallic acid or sample.

Was added 30 μL of sample, 1350 μL of distilled water and 30 μL of Folin-Ciocalteau reagent to an Eppendorf, and then it was subjected to vortex. Next, it rested for 3 minutes before adding 90 μL of sodium carbonate, and then it was set in continuous agitation in a

circular agitator at 18 rpm for 2 hours at 4°C. Finally, the absorbance was measured at 760 nm in a Shimadzu UV-160A.

### 3.1.7. Tannins Content with Prussian Blue

The content of the tannins in the fractions obtained along the study was determined according to the Prussian Blue procedure, using pure tannic acid as standard [4].

The calibration standard curve was obtained using six different concentrations of tannic acid (10, 20, 40, 60, 100 and 150 µg/ mL) and for blank was chosen the same solution without any tannic acid or sample.

Were added 50 µL of sample, 150 µL of distilled water, 150 µL of 0.1 M ferric chloride (in 0.1 M of HCl) and 150 µL of 0.008 M potassium ferrocyanide to an Eppendorf and the absorbance was measured at 605 nm in a Shimadzu UV-160A.

### 3.1.8. Carbohydrates Content Measure

The content of carbohydrates in the fractions obtained along the study was determined with the 3,5-dinitrosalicylic acid (DNS) assay, using monohydrated glucose (dextrose) as standard [5].

The calibration standard curve was obtained using six different volumes of glucose 1.5 mg/mL (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL) and for blank was chosen the same solution without any glucose or sample.

Were added 5 mg of sample and 1 mL of 95-97% $H_2SO_4$ to a test tube, and then it was boiled for 20 minutes. Afterwards, was added to the same tube 1.2 mL of 1M NaOH. The mix obtained was subjected to a vacuum filtration with a 70 MM filter and then it was placed in a 5 mL volumetric flask, which was make up to the mark with distilled water. 1 mL was collected from the previous solution and added to an Eppendorf, along with 1 mL of 3,5-dinitrosalicylic acid (DNS) and 2 mL of distilled water. This solution was boiled again for 5 minutes and then placed in a 25 mL volumetric flask, which was make up to the mark with distilled water. Finally, the absorbance was measured at 540 nm in a Shimadzu UV-160A.

To prepare the DNS solution were added 0.5 g of DNS and 0.8 g of NaOH to 25 mL of heated distilled water and this process was performed in continuous magnetic agitation

until total dissolution of the compounds added. Afterwards, the solution was left until total cooling and then were added 15 g of tartrate and 25 mL of distilled water.

### 3.1.9. Protein Content with Bradford Method

The content of proteins in the fractions obtained along the study was determined according to the Bradford method, using bovine serum albumin (BSA) as standard [6].

The calibration standard curve was obtained using six different volumes of BSA (10, 20, 40, 60, 80 and 100 µL) and for blank was chosen the same solution without any BSA or sample.

Were added 5 µL of sample, 795 µL of distilled water and 200 µL of Bradford reagent to an Eppendorf and the absorbance was measured at 595 nm in a Shimadzu UV-160A.

### 3.1.10. Flavonoid Content

The content of flavonoids in the fractions obtained along the study was determined using a spectrophotometric assay based on aluminium complex formation, using catechin as standard [7].

The calibration standard curve was obtained using six different concentrations of catechin (10, 20, 50, 100, 200 and 500 µg/mL) and for blank was chosen the same solution without any catechin or sample.

Were added 20 µL of sample, 480 µL of distilled water and 300 µL of $NaNO_2$ (5% w/v) to an Eppendorf and the solution was left at room temperature for 5 minutes. Afterwards, were added 30 µL of $AlCl_3$ (10% w/v) and another minute was waited before adding 200 µL of 1M NaOH. The absorbance was measured at 510nm in a Shimadzu UV-160A.

### 3.1.11. Cytotoxicity and Effects on Membrane Integrity Assays

The cytotoxicity was evaluated using general cell viability endpoint MTT reduction (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide) and propidium iodide exclusion assays [8], [9]. The MTT is a yellow water-soluble tetrazolium dye that is reduced to an insoluble-water purple formazan product by living cells. This last product when solubilized in an appropriate solvent can be measured spectrophotometrically, allowing the indirect determination of surviving number cells when exposed to a sample [10].

Cell viability was assessed after 24h of incubation of a spontaneously immortalized human keratinocyte cell line HaCaT (CLS, Germany) with different concentrations of extracts. This assay required three days. In the first day, HaCaT cells were seeded in sterile flat bottom 96 well tissue culture plates (Greiner, Germany), in RPMI 1640 culture medium (Life Technologies, UK), supplemented with 10% Fetal serum bovine, 100 units of penicillin G (sodium salt) and 100 μg of streptomycin sulfate and 2mM L-glutamine (Life Technologies, UK), at a cell density of 2x105 cells/mL 100 microliters per well. Cells were incubated at 37° C and 5% $CO_2$. On the second day, the medium was replaced by fresh medium and the artichoke extract, the artichoke fraction C or their respective formulations were added to six wells per plate. Then, cells were incubated for 24 h, negative control was the culture medium and positive control sodium dodecyl sulfate (SDS) at 1 mg/mL. In the third day, after the time of exposition, the medium was replaced by 0.3 mM propidium iodide in culture medium (stock solution 1.5 mM in DMSO, diluted with culture medium 1:5). Fluorescence was measured (excitation, 485 nm; emission, 590 nm) in microplate reader (FLUOstar Omega, BMGLabtech, Germany), and then, the MTT assay was performed. Medium was replaced by medium containing 0.25 mg/mL MTT. The cells were incubated again for 3h. Afterwards, the medium was removed and the intracellular formazan crystals were solubilized and extracted with 100 μl dimethylsulfoxide (DMSO). After 15 min in continuous agitation at room temperature the absorbance was measured at 570 nm in FLUOstar Omega BMGLabtech Microplate Reader.

The relative cell viability (%) compared to control cells was calculated for the MTT assay using the following equation:

$$\% \text{ cell viability} = \frac{Abs\ sample}{Abs\ control} \times 100$$

Where $Abs_{sample}$ is the absorbance measured for cells treated with artichoke extract or artichoke fraction C formulations and $Abs_{control}$ is the absorbance value obtained for cells incubated with culture medium in the cases of extracts and the absorbance values obtained for cells incubated with its blank in the cases of topical formulations, both measured at 570 nm.

And for propidium iodide assay by:

$$\% \text{ cell viability} = \frac{Fluorescence\ sample}{Fluorescence\ control}$$

where $Fluorescence_{sample}$ is the relative fluorescence unit (URF) values obtained for cells treated with desired samples and $Fluorescence_{control}$ is the URF values obtained for cells incubated with culture medium in the case of extracts and URF values obtained for cells incubated with its blank in the case of topical formulations.

### 3.1.12. Antioxidant Activity

#### 3.1.12.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used for determination of free radical-scavenging activity of the fractions, and the standard utilized was ascorbic acid [11]. DPPH is a stable free radical characterized by an unpaired valence electron at one atom of nitrogen bridge which bears a deep purple color, and when it's neutralized becomes colorless or pale yellow. This method is based on measurement of the loss of DPPH color at 517 nm after reaction with samples [12].

The calibration standard curve was obtained using four different concentrations of the fraction tested (5, 10, 15 and 20 mg/mL) and for blank was chosen the same solution without any sample. Was added 1 mL of DPPH solution and 10 µL of sample to an Eppendorf and it was placed for 30 minutes at room temperature. Then the absorbance was measured at 517 nm in a Shimadzu UV-160A.

The DPPH solution was prepared using 2 mg of pure DPPH and 100 mL of methanol.

The percentages of inhibition of the DPPH free radical were calculated through the following equation:

$$\% \text{ of inhibition} = 100 - (\frac{Af}{Ab} * 100)$$

Where $A_f$ is the absorbance measured for the fraction and $A_b$ is the absorbance measured for the blank. Were realized three independent experiments and the results obtained are expressed as mean ± standard deviation. To calculate the half maximal effective concentration ($EC_{50}$), which is required to scavenge 50% of DPPH free radicals, two approaches were adopted depending on the antioxidant profile observed. A linear

regression was opted for all fractions, with exception of artichoke extract. For this fraction, the $EC_{50}$ value was obtained directly from the profile % of inhibition-concentration obtained.

#### 3.1.12.2. Reactive Oxygen Species (ROS) Production Measurement

The intracellular ROS production can be evaluated through a fluorimeter technique that uses 2,7' dichlorodihydrofluorescein diacetate (H2-DCFDA, Life Technologies, UK). This molecule non-fluorescent is hydrolyzed to 2-7'-dichlorodihydrofluorescein (H2DCF), another non-fluorescent molecule, by the action of intracellular esterases. H2DCF is rapidly oxidized to a highly fluorescent compound (DCF) [13]–[15] in the presence of $H_2O_2$, hydroxyl radicals and several peroxides [16].

In this assay were used HaCaT sub-confluent cells grown in 96 well plates, which were incubated for 30 minutes with 20 μM of H2-DCFDA in dark at 37°C. Later, the medium was removed and fresh medium was added to the cells before being exposed to different concentrations of extracts and ascorbic acid or topical formulations containing the extracts for 1 hour. In the case of the hydrogen peroxide ($H_2O_2$), it was added simultaneously with the samples in study. For the UV assay, three UV-B lamps (Sankyo Denki G8T5E, Kanagawa, Japan) with a peak emission at 312 nm were used as the UV-B source, and measured with a VLX 312 radiometer equipped with a UV-B sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). The cells with the extracts or the topical formulations were irradiated with a UV-B single dose of 26 $mJ/cm^2$ for 15 minutes.

Afterwards, the ROS levels were determined at excitation 485 nm and emission 520 nm wavelengths using a florescence microplate reader (FLUOstar BMGLabtech, Germany) [17]. Data collected from at least 5 replicates is expressed as percentage of reduction of ROS.

### 3.1.13. *In vitro* SPF determination

All natural fractions, including artichoke extract, artichoke fraction C and green coffee oil, were accurately weighed (0.25 g), diluted with ethanol:water solution (1:1), followed by ultrasonication for 5 min and filtered through filter paper (Whatman™ 42). The absorption spectra of samples solution were obtained in the range of 290 to 320 nm (Hitachi U-2001, USA) every 5 nm, using a standard 1 cm quartz cell, and ethanol:water

solution as the blank reagent. Triplicates were made, followed by the application of the Mansur equation:

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE\ (\lambda) \times I\ (\lambda) \times Abs\ (\lambda)$$

where *EE* (λ) is the erythemal effect spectrum; I (λ) is the solar intensity spectrum; Abs (λ) is the absorbance of sunscreen product and CF is the correction factor (=10) [18]. The values of EE x I are constants determined by Sayre [19].

## 3.2. Topical Formulations Development

### 3.2.1. Preparation of an emulsion and a hydrogel

The emulsion was prepared through the combination of different ingredients, being the final formulation recipe in Table 3.1. Parabens sodium salts changed the emulsions colors to pale green, instead of pale beige, so in order get more stable formulations, parabens with no sodium were chosen. In total, three emulsions were prepared: blank cream (BC), cream containing artichoke extract (AC) and cream containing artichoke fraction C (CC).

Table 3.1 - Percentage of ingredients in artichoke extract and artichoke fraction C emulsions.

| Ingredients | Quantitative Composition (%, w/w) |
|---|---|
| Polyglyceryl-3 Dicitrate/Stearate (Tego Care PSC3®) | 3 |
| Cetearyl Alcohol (Tego Alkanol 1618®) | 7 |
| Liquid Paraffin | 2.5 |
| Decyl Oleate (Tegosoft Do®) | 4.5 |
| Almond Oil | 5 |
| Glycerin | 5 |
| Purified Water | 71.8 |
| Methylparaben (Nipagin®) | 0.18 |
| Propylparaben (Nipazol®) | 0.02 |
| Dry Artichoke Extract/ Dry Artichoke Fraction C | 1 |

The process to prepared the emulsion involved the following steps:

I. Adding the oily compounds (polyglyceryl-3 dicitrate/stearate, cetearyl alcohol, liquid paraffin, decyl oleate and almond oil) to a bowl and the non-oily compounds (glycerin and 70% of the purified water) to another bowl and place both bowls in a water bath at 70-80°C until total homogenization;
II. Dissolving the extract in the remain purified water (around 30%) and reserve it;
III. Verting the oily ingredients to the bowl containing the non-oily ingredients;
IV. Taking the mix to ultra-turrax during 2-3 minutes;
V. Adding the parabens to the mix while it is still warm;
VI. Stirring the mix until it's cold;
VII. Adding the dissolved extract and then agitating the mix until it is homogeneous.

The ingredients used to prepare the hydrogel are shown in Table 3.2. The polymer chosen to prepare the hydrogel was a carbomer, Carbopol 940®, which is a cross-linked polyacrylate polymer, that confers high viscosity, high suspending ability and high clarity to the formulation. The preservatives chosen were methyl and propyl parabens with no sodium. In total, three gels were prepared: blank gel (BG), gel containing artichoke extract (AG) and gel containing artichoke fraction C (CG).

Table 3.2 - Percentage of ingredients in artichoke extract and artichoke fraction C hydrogels.

| **Ingredients** | **Quantitative Composition (%, w/w)** |
|---|---|
| Purified Water | 86.48 |
| Carbomer (Carbopol 940®) | 1.12 |
| Glycerin | 11.2 |
| Methylparaben (Nipagin®) | 0.18 |
| Propylparaben (Nipazol®) | 0.02 |
| Dry Artichoke Extract/ Dry Artichoke Fraction C | 1 |

The process to develop the hydrogel involved the following steps:

I. Adding all ingredients to a bowl, except the dry extract and some of the purified water;
II. Dissolving the extract in the remain purified water and reserve it;

III. Adding the dissolved extract to the bowl;
IV. Leaving the mix for 30 minutes at room temperature;
V. Taking the mix to ultra-turrax during 2-3 minutes;
VI. Adding sodium hydroxide to the mix until it gained the desired consistency.

A blank for each formulation were also prepared to be used as control standards in the assays using the emulsions and gels.

### 3.2.2. Formulations Rheology

All formulations prepared, including their blanks, were analysed relatively to its consistency. Therefore, its viscosity was measured with a rotational digital viscometer Brookfield DV II and small sample adapter (SSA) – Brookfield Engineering Laboratories, EUA. The viscosity of the two formulations prepared, emulsion and hydrogel, were very distinct, so two spindles were used. For the emulsions was used the spindle nº 27 and for the hydrogels the spindle nº 7. Approximately 25 mL of formulation were placed in the recipient of the viscometer, and it was subdued to a continuous shear rate sweep (from 0,6 to 122 $s^{-1}$), keeping each shear rate for 15 seconds.

### 3.2.3. pH Measurement

The pH of each formulation were measured with the pH meter InoLab 730 WTW, at room temperature. At least three measures were performed for each emulsion and each gel, and the data were collected after stabilization of value on the equipment.

### 3.2.4. Microbiological Control

The microbiological stability assessment was performed according to the ISO 16212:2008, ISO 21149:2006 and ISO 21148:2005 [20]–[22].

## 3.3. In Vivo Studies Analysis

### 3.3.1. Human Repeat Insult Patch Testing (HRIPT)

Marzully and Maibach HRIPT protocol [23] was used to performed a safety evaluation. For this study were chosen 50 healthy volunteers that were informed about the procedure and had signed the informed written consent. Volunteers with dermatological or other

medical or physical problems, and pregnant and nursing women were excluded from the study. The induction period of a possible allergic response lasted three weeks, in which a series of 9 patches (Finn Chamber standard) were performed. Each occlusive patch contained 20 mg of the formulation and was applied on the left side of the back. After 48 hours, the patch was removed and was performed a skin evaluation, which was followed by the application of a new patch. Reactions after patching were measured according to the recommendations of the International Contact Dermatitis Research Group [24].

Induction period was followed by a two weeks' rest period, in which wasn't applied the tested product. Afterwards, challenge period started and new patches were applied again in the same way as in the induction period, but this time were applied in the right side of the back. After a period of 48 hours the patches were removed and skin evaluations were realized to observe possible skin reactions at 48, 72 and 96 hours after patching using again the score method recommended by the International Contact Dermatitis Research Group.

This protocol was approved by the local Ethical Committee and respected the Helsinki Declaration and the AFSSAPS regulations on performed HRIPT studies on cosmetic products. This study was supervised by a dermatologist who participated in the skin evaluations in order to observe possible irritation/allergic reactions to the tested formulations.

### 3.3.2. Assessment of the protective effect against oxidative stress after UV radiation by chromameter evaluation

In the first day, three patches were applied in the forearm area (left or right) in each volunteer, two contained the formulations and the other one was the control, in which no product was applied. The patches remained 24 hours, and after this period, a solution of β-carotene was applied in the same area of the forearm. A negative control area with only β-carotene was also used. After 15 min of β-carotene application, the color was measured in all the areas. The areas were then irradiated with a UVA lamp and the color was measured again. A Minolta Chromameter CR-400 (Minolta, Japan) was used to obtain the b* color. The same area was evaluated at the first day (D0) and then after 28 days (D28) using the same procedure. Between D0 and D28, the volunteers applied the topical products in the areas indicated by the principal investigator.

## 3.4. Statistical Analysis

Data was expressed as mean and standard deviation (mean ± SD) of experiments. Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Tukey–Kramer multiple comparison test (GraphPad PRISM 7® software, USA), was used to compare the significance of the difference between the groups, a $P < 0.05$ was accepted as significant.

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# 4. Results and Discussion

## 4.1. Artichoke Extract

The artichoke extract was chosen to be the main subject in this study due to its antioxidant properties and it was obtained through an infusion, followed by a lyophilization. Its chromatogram is showed in Figure 4.1.

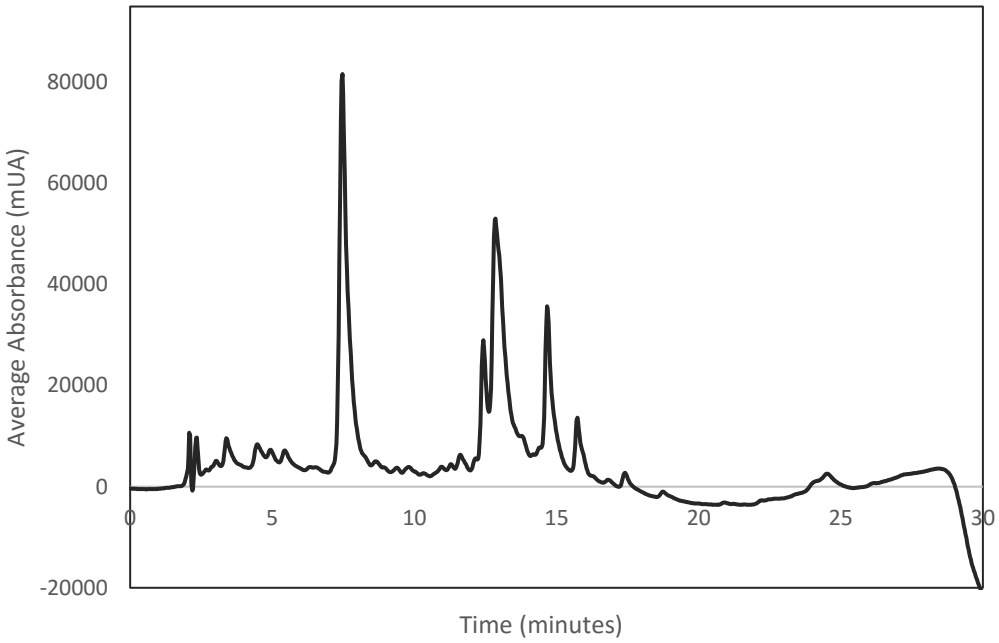


Figure 4.1 - Example of chromatogram of artichoke infusion (1mg/mL).

## 4.2. Purification of Artichoke Extract

In order to purify the artichoke extract and to improve its properties, including antioxidant activity, two different purification approaches were addopted: an acidic digestion with gastric juice followed by a 24 hours dialysis, and a mucilage precipitation method. In this last one, the importance of acetic acid in the procedure was also investigated. The purification schematics is presented in Figure 4.2.

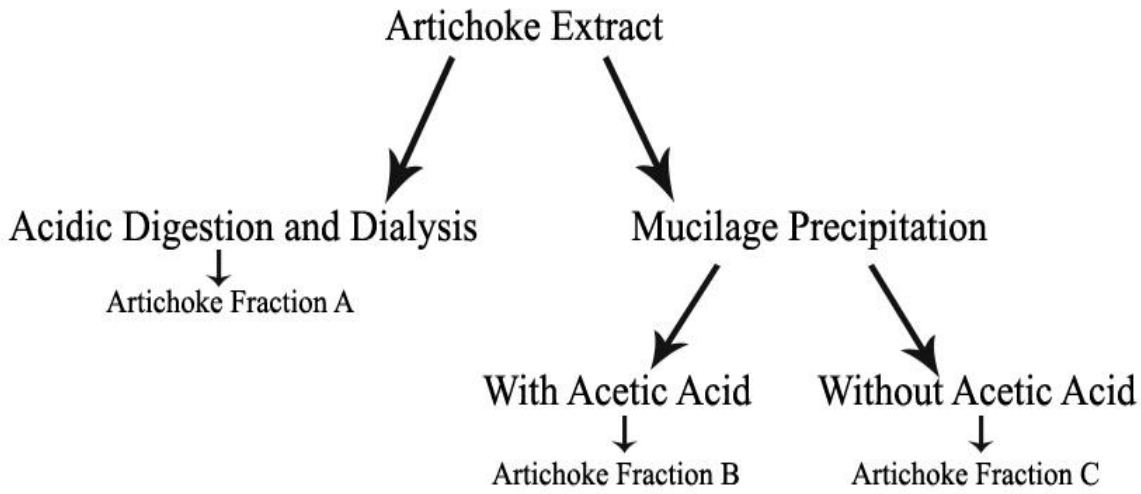


Figure 4.2 - Scheme of the purification strategies adopted to purify the artichoke extract.

### 4.2.1. Acidic Digestion & Dialysis (Artichoke Fraction A)

Tea infusions when ingested are exposed to gastric juice, and consequently to a complex system of enzymes and to a highly acidic pH (1.0-2.5) [1]. Pepsin is one of those enzymes and its optimum pH range is 1.5-2.5, which means that it has its maximum activity inside the stomach [2]. It can cleave the linkages between mucilage and phenolic compounds, enhancing the extraction of polyphenols from the artichoke extract during dialysis. Therefore, in order to maximize the extraction of polyphenols compounds from the artichoke extract, a dialysis with a previous acidic digestion of artichoke extract was made.

To follow the progress of dialysis, aliquots of the permeate were collected along the assay. The permeate – the outer water where the retentate is placed - was exchanged three times to avoid saturation (3, 6 and 22 hour), which would probably slow down the polyphenols extraction. Over time was observed a change of coloration in the permeate, being the earlier samples colorless and the latest pale brown. This indicated that probably there was occurring an extraction from the artichoke extract to the permeate. This purified fraction will be called Artichoke Fraction A.

Phenolic compounds present in each aliquot were measured through a well-established method of polyphenols quantification. It's important to mention that the outer waters were exchanged after the hour 3, 6 and 22, so the values present in

Table 4.1 were not cumulative in the water. The maximum polyphenols mass was extracted after three hours of dialysis, meaning that a considerable quantity of polyphenols present in the artichoke extract were transferred from inside the dialysis membrane to the permeate in the first hours. In the following hours of dialysis, the rate of extraction decreased substantially, for example the mass of polyphenols extracted in 16 hours – between the collection of the samples of hours 6 and 22 – was only 72 µg (

Table 4.1; Figure 4.3). The total polyphenols extracted during this process was the sum of all polyphenols present in the outer waters collected along the process (hours 3, 6, 22 and 24).

Table 4.1 - Quantification of polyphenols in artichoke fraction A after 3, 6, 22 and 24 hours of dialysis.

| Time (hour) | Quantification of polyphenols (µg) |
|---|---|
| 3 | 295 |
| 6 | 100 |
| 22 | 72 |
| 24 | 3 |

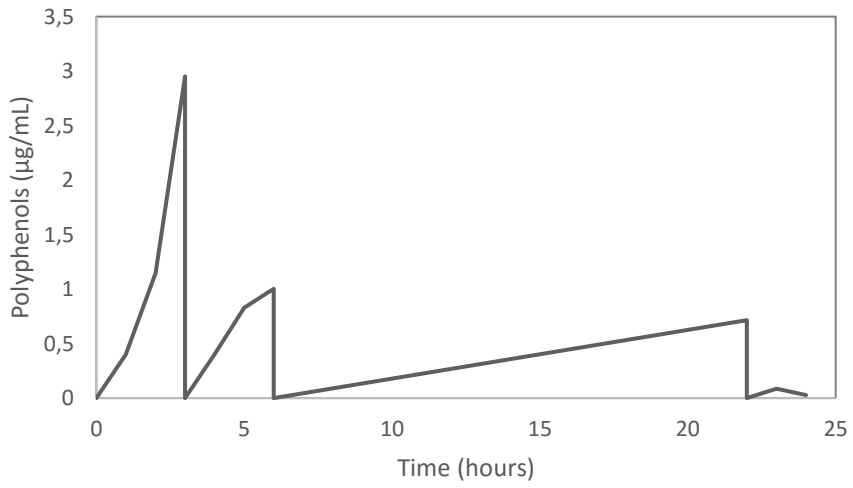


Figure 4.3 - Quantification of polyphenols in each aliquot collected from the artichoke fraction A during the 24 hours of dialysis.

The HPLC-DAD chromatograms of the artichoke extract and the retentate – part of the extract that does not pass through the dialysis membrane – showed the presence of the same compounds in both fractions, since there are traces of every compound present in the artichoke extract in the retentate chromatogram (Figure 4.4). The same occurred in the HPLC-DAD chromatograms of the retentate and the artichoke fraction A (Figure 4.5).

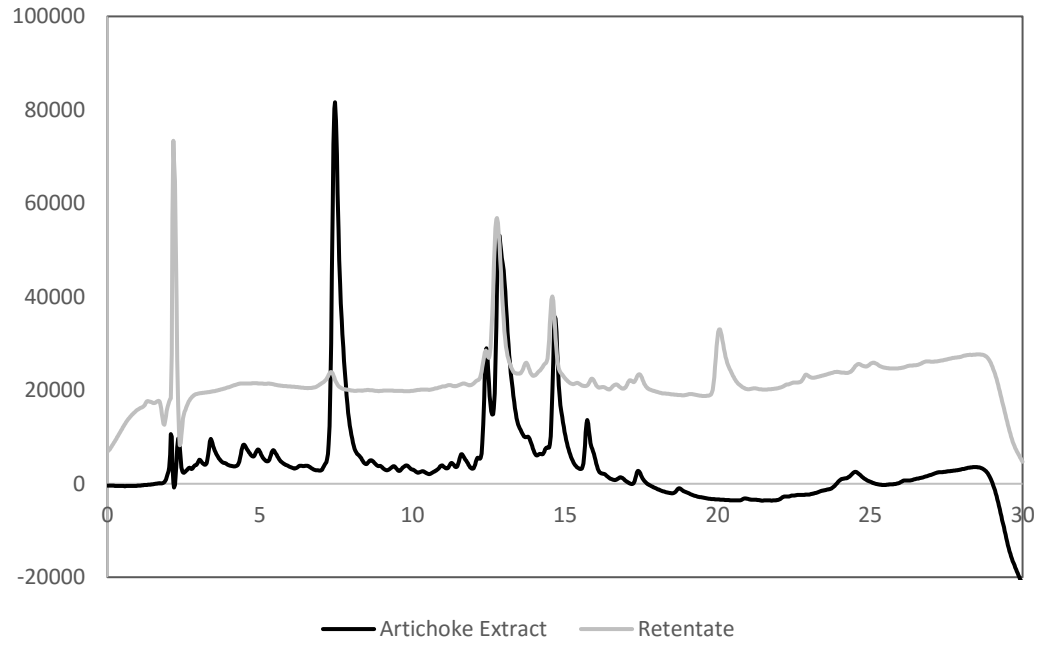


Figure 4.4 - Yield of compounds identified for artichoke extract and retentate obtained after dialysis by HPLC-DAD.

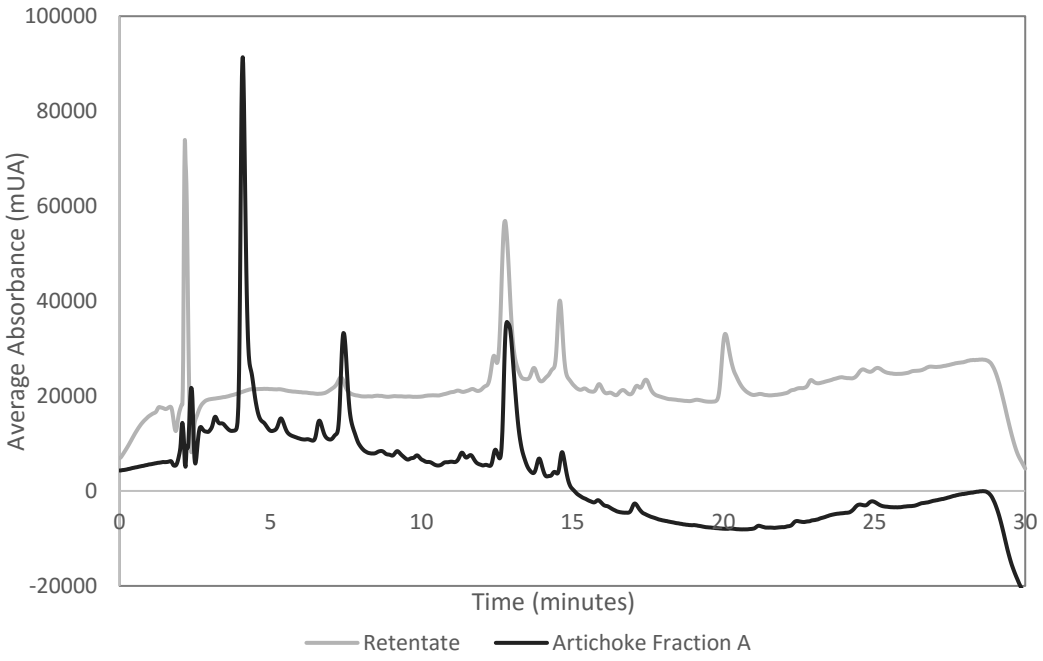


Figure 4.5 - Yield of compounds indentified for retentate and artichoke fraction A, both obtained after dialysis, by HPLC-DAD.

A control sample constituted by 50% water and 50% gastric juice was analyzed by HPLC-DAD in order to compare with the rest of the chromatograms obtained from dialysis samples. In Figure 4.6, the retentate and digested artichoke extract (artichoke extract after the acidic digestion with gastric juice) chromatograms showed a peak at minute 25.2 that didn't exist in the artichoke extract spectrum. However, this peak was also present in the control chromatogram, which suggests that it corresponds to the pepsin enzyme. This implies that the enzyme is not transferred from the retentate to the artichoke fraction A through the dialysis membrane, which complies with pepsin molecular weight (≈ 36 000 Da) and the molecular weight cutoff of the membrane pores (10 000 Da).

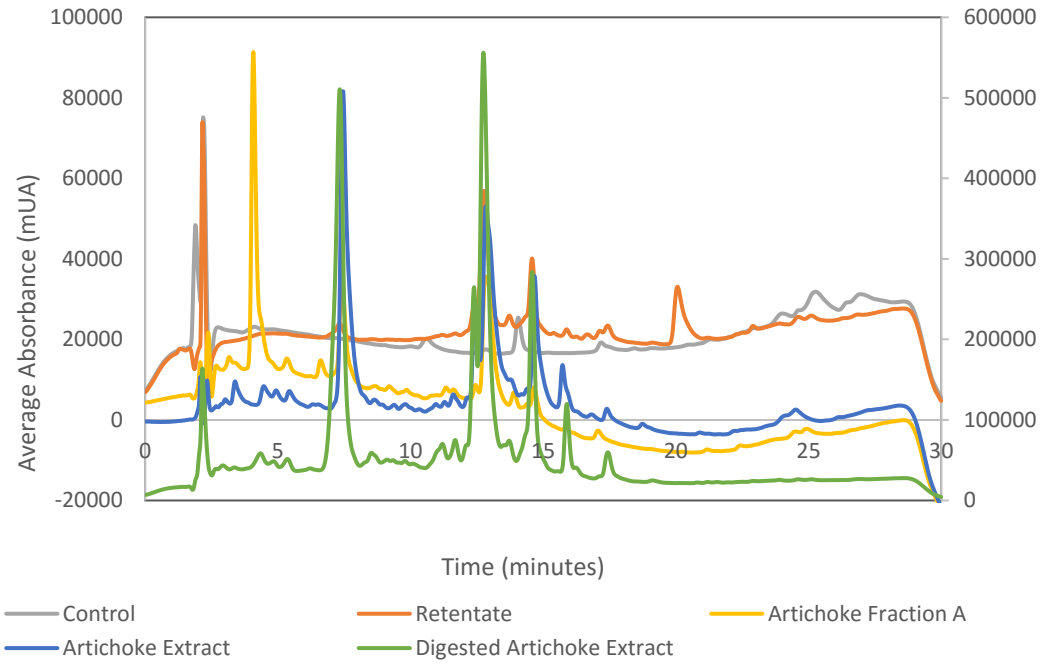


Figure 4.6 - Yields of compounds identified for the control sample, the retentate, the artichoke fraction A, the artichoke extract and the digested artichoke extract by HPLC-DAD. Control sample is 50% gastric juice and 50% water. Digested artichoke extract chromatogram is represented in a secondary vertical axis.

### 4.2.2. Mucilage Precipitation (Artichoke Fractions B and C)

Previous studies demonstrated the potential of polyphenolic-polysaccharide glycoconjugates isolated from medicinal plants of Asteraceae family as antioxidants, once they exhibited the capacity to protect proteins and lipids present in human plasma against oxidative stress caused by γ-radiation [3]. However, Kim *et al.* [4] showed that mucilage removal through an enzymatic hydrolysis from cactus cladodes resulted in an increase of phenolic compounds in the extract, and consequently in the improvement of its radical scavenging activity. Therefore, in order to remove the mucilage present in the

artichoke extract, a method found in literature was used [5]. This method had as purpose the improvement of the phenolic content of artichoke extract and consequently, its antioxidant activity.

Initially, it was tested whether the presence of acetic acid affected the mucilage precipitation of the artichoke extract, in terms of phenolic compounds and antioxidant activity. Both assays were realized, one using acetic acid and the other without acetic acid, remaining the rest of the procedure equal. The supernatant obtained from the method using acetic acid will be called Artichoke Fraction B and the supernatant from the method without acetic acid will be named Artichoke Fraction C.

## 4.3. Extracts Chemical Analysis

### 4.3.1. Artichoke Extract

#### 4.3.1.1. HPLC-DAD Analysis

Previous studies showed the presence of chlorogenic acid (5-caffeoylquinic acid), cynarin (1,3-di-O-caffeoylquinic acid) and cynaroside (luteolin-7-O-glucoside) in the artichoke extract [6]. Therefore, in order to investigate the presence of these compounds in the artichoke infusion previously prepared, a HPLC-DAD analysis was performed, being the chromatogram obtained in Figure 4.7.

The retention times of chlorogenic acid, cynarin and cynaroside were approximately 7.38, 12.4 and 12.65 min, respectively. So, the peak 1 indicated in Figure 4.7 corresponds to chlorogenic acid, the peak 2 to cynarin and the peak 3 to cynaroside.

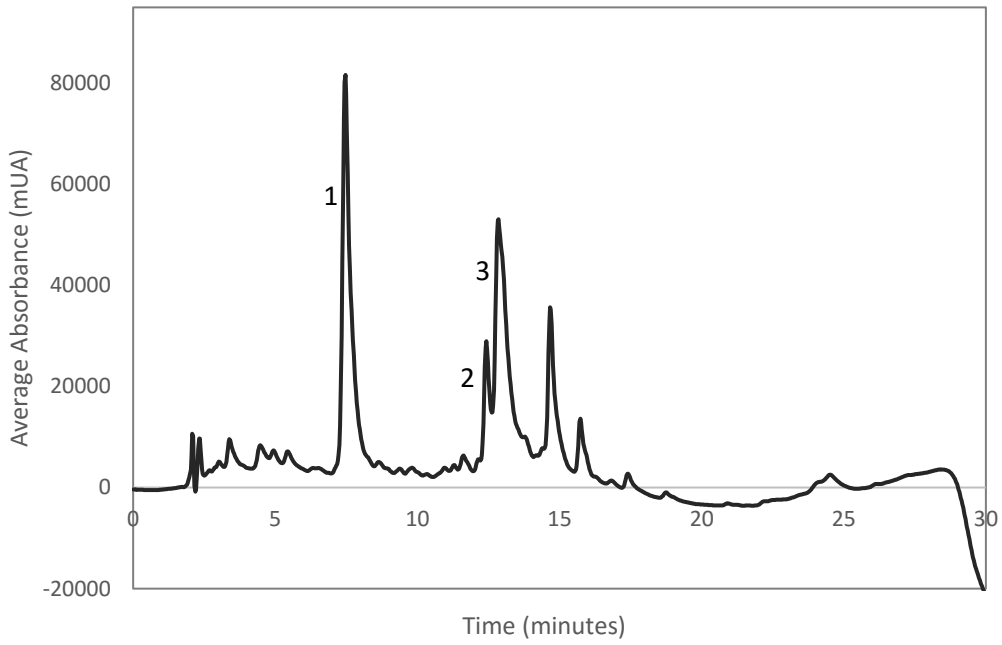


Figure 4.7 – Example of chromatogram of artichoke infusion (1mg/mL).

To measure these antioxidants in the artichoke infusion were performed several HPLC-DAD analyses of chlorogenic acid, cynarin and cynaroside with well-known concentrations to create HPLC-DAD calibration curves, being the results in Table 4.2. Cynarin showed to be the most abundant antioxidant in the artichoke extract with aproximally 83 μg/ mg of artichoke extract, and chlorogenic acid revealed to be also in high levels with aproximally 56 μg/ mg of artichoke extract. This outcome is in accord with the literature, where chlorogenic acid and cynarin are showed to be the mainly compounds of *Cynara C. Scolymus* [7]. The flavone cynaroside is present in a smaller amount when compared with the two caffeoylquinic acids, with only 7 μg/mg of artichoke extract. Therefore, artichoke extract showed to be rich in phenolic compounds and flavones, which can explain its antioxidant activity.

Table 4.2 – Total content of chlorogenic acid, cynaroside and cynarin in the artichoke extract.

| | **Total Content (μg/mg of artichoke extract)** |
|---|---|
| Chlorogenic Acid | 56 |
| Cynaroside | 7 |
| Cynarin | 83 |

#### 4.3.1.2. McCleary Method

In the mucilage precipitation method was observed a smaller amount of precipitate than it was expected, so was also realized the McCleary method. This method consists in two successive enzymatic digestions, the first with gastric juice and the second with pancreatic juice, and it was used to quantify the total dietary fibers present in the artichoke extract in order to characterize it. The artichoke fraction C was used in the beginning of this method. Its schematic is represented in Figure 4.8.

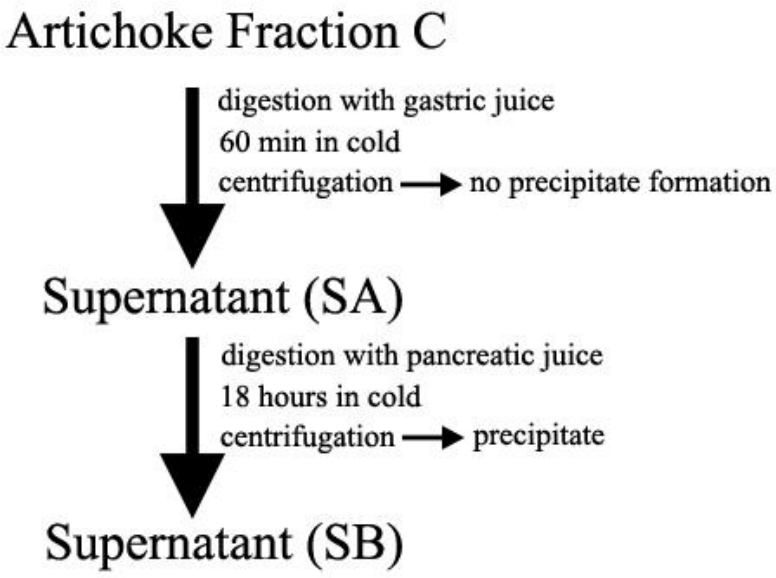


Figure 4.8 - Scheme of the McCleary method.

In order to follow the process, aliquots of the supernatants and precipitate formed were collected and posteriorly analyzed by HPLC-DAD. The chromatograms of the artichoke fraction C and the supernatants obtain during this method are represented in Figure 4.9, and some differences are spotted between them, like the peaks intensities as well as the appearing of new peaks and the disappearing of old ones. These results suggest that during the process, and due to the digestions by gastric and pancreatic juices, there are alterations in the artichoke extract, which are expected to lead to a more purified extract.

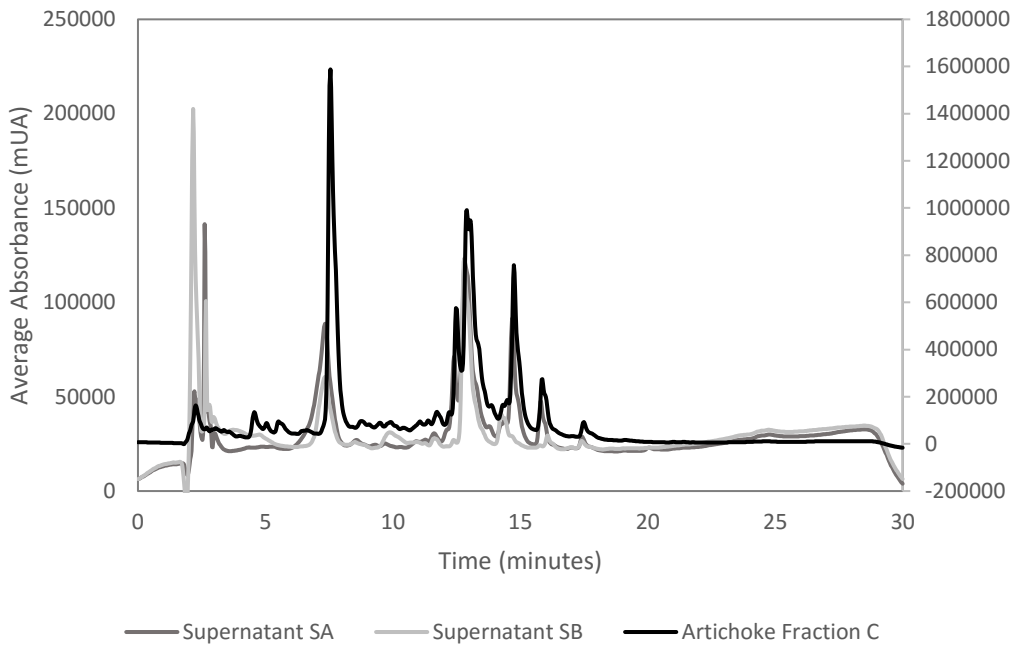


Figure 4.9 - HPLC-DAD analysis of the artichoke fraction C (15 mg/ mL), and the supernatants SA (1,5 mg/ mL) and SB (1,3 mg/ mL). The artichoke fraction C is in a seconday axis (right one).

In the gastric juice digestion realized in the first purification method, dialysis, was observed the formation of a thin brown layer inside the dialysis membrane containing the artichoke extract, just like the layer observed also in the previous mucilage precipitation method, which means that the digestion of the extract using gastric juice results in the precipitation of mucilage. However, in this method, after the gastric juice digestion, wasn't observed any layer and consequently, wasn't formed any precipitate. This could be due to the previous mucilage precipitation method that resulted in the artichoke fraction C, which perhaps precipitated the all mucilage susceptible to the gastric juice enzymes. A closer look in the chromatograms of the artichoke fraction C and the supernatant SA, represented in Figure 4.10, shows that there are no significant differences between them, with exception of the peaks intensities. Therefore, this digestion didn't affect the composition of the artichoke extract but according with the results showed in Table 4.3, there was an increase in the total content of phenolic compounds in the SA supernatant when compared with the content of the artichoke fraction C.

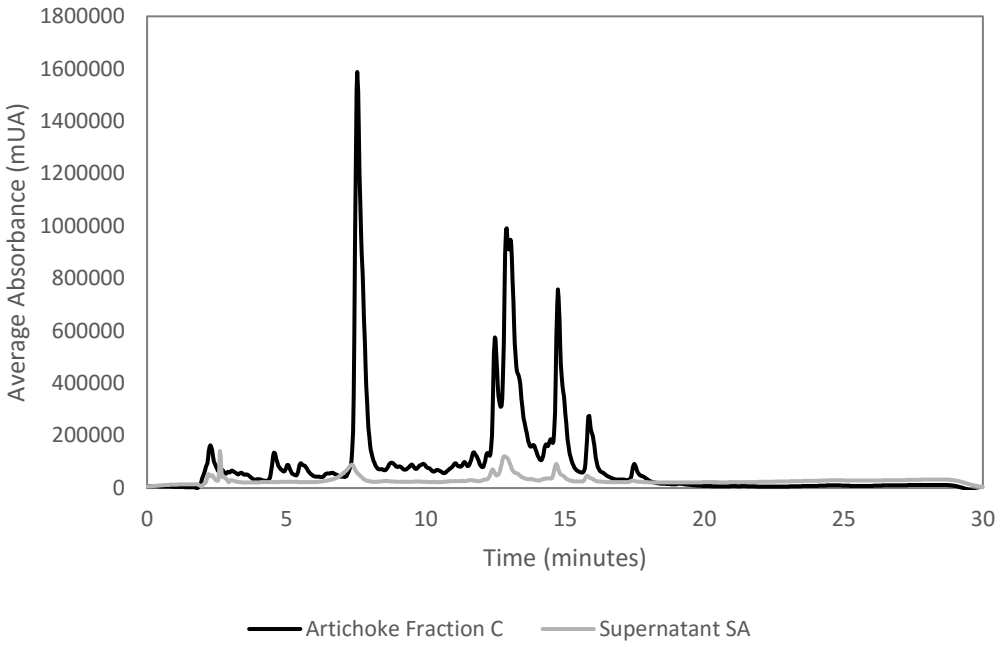


Figure 4.10 - HPLC-DAD analysis of the artichoke fraction C (15 mg/ mL) and the supernatant SA (1,5 mg/ mL).

Table 4.3 - Total content of total phenols present in the artichoke fraction C and in the supernatants SA and SB. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **Phenols (µg/ mg artichoke extract)** |
|---|---|
| Artichoke Fraction C | 25 ± 0.78 |
| SA supernatant | 30 ± 1.98 |
| SB supernatant | 31 ± 3.68 |

The chromatograms of the SA and SB supernatants shown in Figure 4.11 have some differences between them, including the appearing of the peak 1 and the nearly disappearing of the peak 2 in the SB chromatogram. These alterations are probably due to the pancreatic digestion, which results in the alteration of the artichoke extract composition. Bouayed *et al.* [8] studied the influence of intestinal digestion, which includes pancreatic juice, in the polyphenols of selected apple varieties. Changes in the polyphenolic profiles during intestinal digestion were observed and a considerable loss of polyphenols occurred. Another study reported the change of polyphenols after pancreatic digestion, which was traduced by the decrease of anthocyanins, flavonols, flavan-3-ols and neochlorogenic acid, and the increase of chlorogenic acid [9]. However, in this study was observed a slightly increase of total phenols after gastric and pancreatic digestion, being the results in Table 4.3.

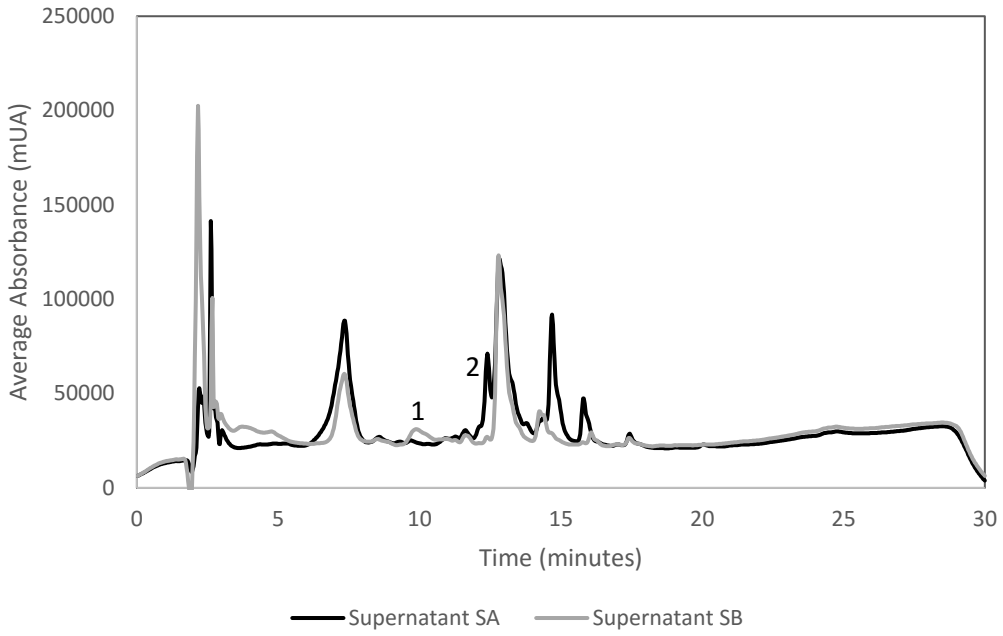


Figure 4.11 - HPLC-DAD analysis of the SA (1,5 mg/ mL) and SB (1,3 mg/ mL) supernatants.

The HPLC-DAD analysis of the SB supernatant, the precipitate formed and the pancreatic juice, which chromatograms are presented in Figure 4.12 and Figure 4.13, suggests that a fraction of the pancreatic juice remained in the supernatant and another fraction precipitated with the mucilage. The peak 1 market in Figure 4.12, already marked before in the Figure 4.11, appears in the SB supernatant and in the pancreatic juice, but not in the SA supernatant, which indicates that this peak corresponds to a compound of the pancreatic juice, meaning that a part of it remained in the supernatant. On the other hand, the peaks 1 and 2 market in Figure 4.13 represent the compounds present either in the precipitate or in the pancreatic juice, indicating that a fraction of the pancreatic juice precipitates along with the mucilage. The precipitation of pancreatic juice compounds can possible be explained by the interactions between proteins and polysaccharides through an electrostatic complex or micellar structures formation [10], which perhaps are responsible for dragging the enzymes present in the solution, like pepsin and pancreatin, along with the polysaccharides.

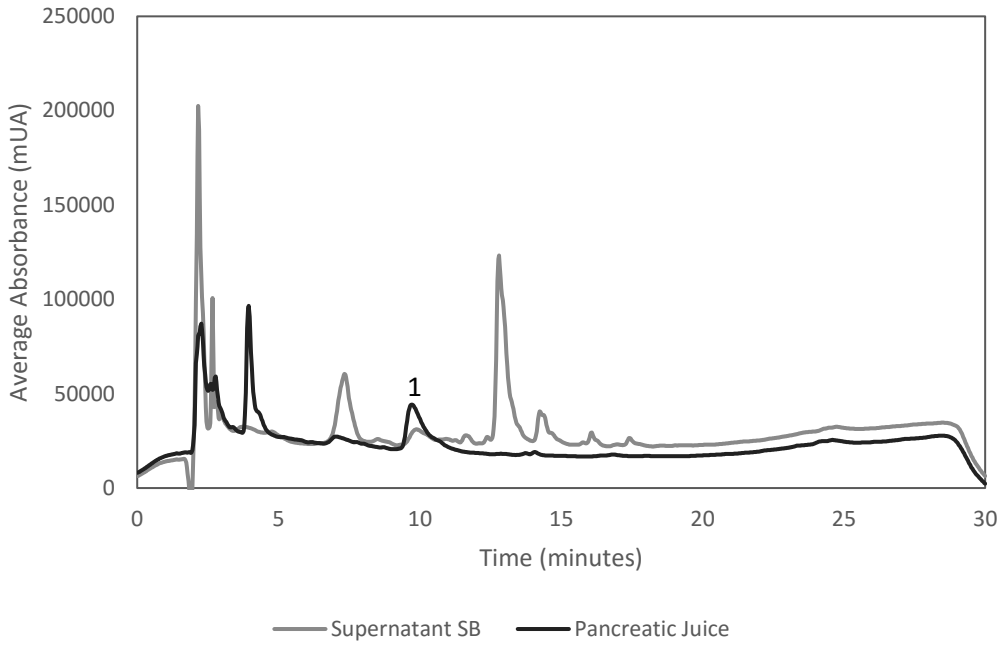


Figure 4.12 - HPLC-DAD analysis of the SB supernatant (1,3 mg/ mL) and the pancreatic juice (2,5 mg/ mL).

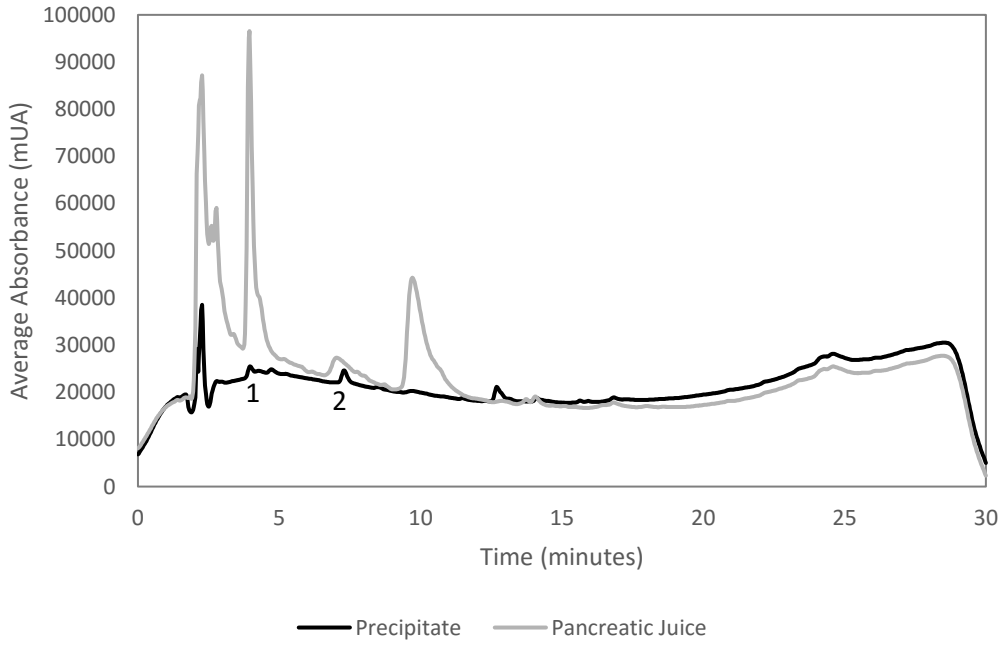


Figure 4.13 - HPLC-DAD analysis of the precipitate obtained and the pancreatic juice (2,5 mg/ mL).

The precipitate formed in the last step of this method had a total mass of 18.2 mg. But to assume that all of it corresponds only to mucilage, a calculation of the mass of pancreatic juice present in it was needed. The peaks 1 and 2 of the precipitate chromatogram market in Figure 4.13 corresponds to 0.22 mg and 0.93 mg of pancreatic juice. Therefore, from

the total mass of the precipitate obtained, 17.1 mg corresponds to dietary fibers. Combining this result with the previous result of the mucilage precipitation method applied before to the artichoke fraction C (13.7 ± 2.1 %), the artichoke extract has 62.8 ± 1.9 % of mucilage.

Also, the precipitate formed had a low solubility, and not even with sonication and vortexing methods was possible to dissolve it in distilled water, which suggests that the majority of the precipitate obtained from the McCleary method were insoluble dietary fibers.

#### 4.3.1.3. Global composition

The artichoke is a higher plant, which means that its cells walls are made up of aproximally 90% carbohydrates and 10% proteins, and it has in its constitution phenolic compounds and dietary fibers as well [11], [12]. Therefore, carbohydrates, proteins, polyphenols and soluble and insoluble fibers were measure in the artichoke extract to proceed to its characterization, being the results in Table 4.4. A total of 66.7% of the artichoke extract was characterized, being the soluble and insoluble fibers the most abundant compound of the *Cynara C.* var. *Scolymus* infusion, corresponding to 62.8% of it. The content of proteins showed to be the lowest, only 0.002%, which can be a result of the thermal process involved in the preparation of the artichoke infusion. Other studies, also reported a decrease of protein levels in vegetables after being boiled [13]. The levels of carbohydrates and phenolic compounds correspond to 0.82% and 3.06% of the artichoke extract, respectively.

Table 4.4 - Quantity of carbohydrates, proteins, phenols and soluble and insoluble fibers present in a total of 80 mg of artichoke extract. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | Carbohydrates | Proteins | Phenols | Fibers | Total |
|---|---|---|---|---|---|
| **Mass (µg)** | 661 ± 250 | 1.59 ± 0.92 | 2 452 ± 80.2 | 50 266 | 53 381 |
| **%** | 0.82 ± 0.31 | 0.002 ± 0.001 | 3.06 ± 0.10 | 62.8 | 66.7 |

Petacci *et al.* [14] quantified the polyphenols content of 12 Asteraceae species, including *Ageratum Fastigiatum, Chromolaena Chaseae, Eremanthus Elaeagnus, Lepidaploa Lilacina, Lepidaploa Rufogrisea, Lychnophora Sp., Lychnophora Ericoides, Lychnophora Ramosissima, Mikania Nummularia, Trichogonia Villosa, Trixis Glutinosa*

and *Echinocoryne Holosericea*, and their polyphenols content ranged between 32.65 ± 0.70 and 147.97 ± 2.66 mg per 100 g of extract. On the other side, in this study the *Cynara Cardunculus Scolymus*, specie also from the *Asteraceae* family, showed 306 mg per 100g of extract, being a richer source of polyphenols.

### 4.3.2. Artichoke Fraction A

To evaluate the efficiency of polyphenols extraction from artichoke extract to the Artichoke Fraction A, samples of retentate and artichoke fraction A were collected before and after dialysis in order to perform a few quantifications.

Analysis of the total phenols and tannins in all fractions showed that dialysis managed to extract phenols and tannins from the inside of tubing dialysis to the artichoke fraction A, being the results in Table 4.5.

Table 4.5 - Concentration of total phenols and tannins in artichoke extract, digested artichoke extract before dialysis and in the retentate and artichoke fraction A after dialysis. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **Concentration (µg/mg of artichoke extract)** | | | |
|---|---|---|---|---|
| | Artichoke Extract | Digested artichoke extract | Retentate | Artichoke Fraction A |
| **Phenols** | 31 ± 1.0 | 16 ± 5 | 3 ± 0.5 | 35 ± 10 |
| **Tannins** | 0.05 ± 0.002 | 0.02 ± 0.001 | 0.002 ± 0.0003 | 0.03 ± 0.009 |

Previous studies using chokeberry reported no substantial effect caused by gastric digestion on any of the major phenolic compounds, including anthocyanins, flavonols, flavan-3-ols and caffeic acid derivatives [9]. However, in this study was observed a decrease of polyphenols concentration present in the artichoke extract from 31 ± 1.0 to 16 ± 5 µg/mg of artichoke extract after the gastric digestion. Bouayed *et al.* [8] also reported a decrease of polyphenols in four apple varieties after gastric digestion when compared with their original content. Polyphenols and proteins, including enzymes like pepsin, can be bonded through reversible or irreversible non-covalent interactions, such as hydrogen bonding, hydrophobic and ionic interactions [15]. Therefore, the decrease observed may be due to these interactions.

Afterwards, in the dialysis process was observed a decrease of polyphenols and tannins concentration in the retentate compared to the initial levels in the digested artichoke

extract (from 16 ± 5 to 3 ± 0.5 µg/mg of artichoke extract in total phenols; from 0.02 ± 0.001 to 0.002 ± 0.0003 µg/mg of artichoke extract in tannins), and the opposite occurred in the artichoke fraction A, which levels increased (from 0 to 35 ± 10 µg/mg of artichoke extract in total phenols and to 0.03 µg/mg of artichoke extract in tannins). Notice that the levels of phenols and tannins are higher in artichoke fraction A than in the digested artichoke extract, suggesting that the interaction between polyphenols and proteins, such as pepsin, was reversible. The same happened between artichoke fraction A and artichoke extract, which suggests that a purification occurred, increasing polyphenols values per mg of artichoke extract.

A quantification of chlorogenic acid, cynaroside and cynarin in the artichoke extract, digested artichoke extract, retentate and artichoke fraction A was also performed. These results are shown in Table 4.6. After acidic digestion, a decrease of chlorogenic acid and cynarin in the artichoke extract occurred, from 56 to 48 µg/mg of extract and from 83 to 40 µg/mg of extract, respectively. On the other hand, an increase of cynaroside concentration was observed, from 7 to 33 µg/mg of extract. After dialysis, was observed a decrease of all components between the digested artichoke extract and the retentate, from 48 to 15 µg/mg of extract in chlorogenic acid, 33 to 3 µg/mg of extract in cynaroside and 40 to 28 µg/mg of extract in cynarin, which suggested that the transferring of these compounds occurred to the artichoke fraction A. This can be confirmed through the comparison of their values between the retentate and the artichoke fraction A, once occurred an increase of all in artichoke fraction A.

Table 4.6 - Concentration of chlorogenic acid, cynarin and luteolin in digested artichoke extract before dialysis and in the retentate and artichoke fraction A after dialysis.

| | **Concentration (µg/mg of extract)** | | | |
|---|---|---|---|---|
| | Artichoke Extract | Digested artichoke extract | Retentate | Artichoke Fraction A |
| Chlorogenic Acid | 56 | 48 | 15 | 42 |
| Cynaroside | 7 | 33 | 3 | 7 |
| Cynarin | 83 | 40 | 28 | 71 |

Considering all results obtained, presented in Table 4.5 and Table 4.6, it is possible to conclude that this method was able to extract polyphenols, including chlorogenic acid, cynarin and cynaroside, from the artichoke infusion, which resulted in a purified fraction (artichoke fraction A).

The artichoke fraction A was lyophilized and a total mass of 572.8 mg was recuperated, but according to calculations, only 1.32 mg of polyphenols and tannins were extracted to this fraction. Therefore, the dialysis tubing allowed other artichoke compounds to pass to the permeate besides the polyphenols. These may be polysaccharides, so a mucilage quantification was performed and the results demonstrate that 22% of the lyophilized artichoke fraction A was mucilage. The same analysis was made for the lyophilized retentate and it showed that 33% were mucilage. The existence of mucilage inside and outside of the tubing dialysis probably happens due to size variances of the polysaccharides, being the molecular weight cutoff of 10 000 Da, meaning that only the molecules with a smaller size than 10 000 Da can pass through the pores to the artichoke fraction A.

Initially were added 550 mg of artichoke extract, 35 mg of pepsin and 22 mg of sodium chloride, these last ones were part of gastric juice, to the dialysis membrane, resulting in a total mass of 607 mg. However, 43.6 mg of retentate and 572.8 mg of artichoke fraction A were recuperated after lyophilization, which equals 616 mg. Comparison of the initial mass with the recuperated showed a discrepancy of 9 mg. Lyophilization results in the absorption of 5-10% (w/w) of water by the dried mixture, so this difference observed maybe was due to its water content that wasn't lyophilized [16].

### 4.3.3. Artichoke Fraction B and C

As showed in Figure 4.14, the chromatograms of both supernatants, are very similar, being only notable a slight difference within the peaks intensities. The same happened with the precipitates chromatograms as it is possible to observe in Figure 4.15. Therefore, the absence of acetic acid in the method used to precipitate the mucilage present in the artichoke extract doesn't affect the composition of the supernatant and the precipitate.

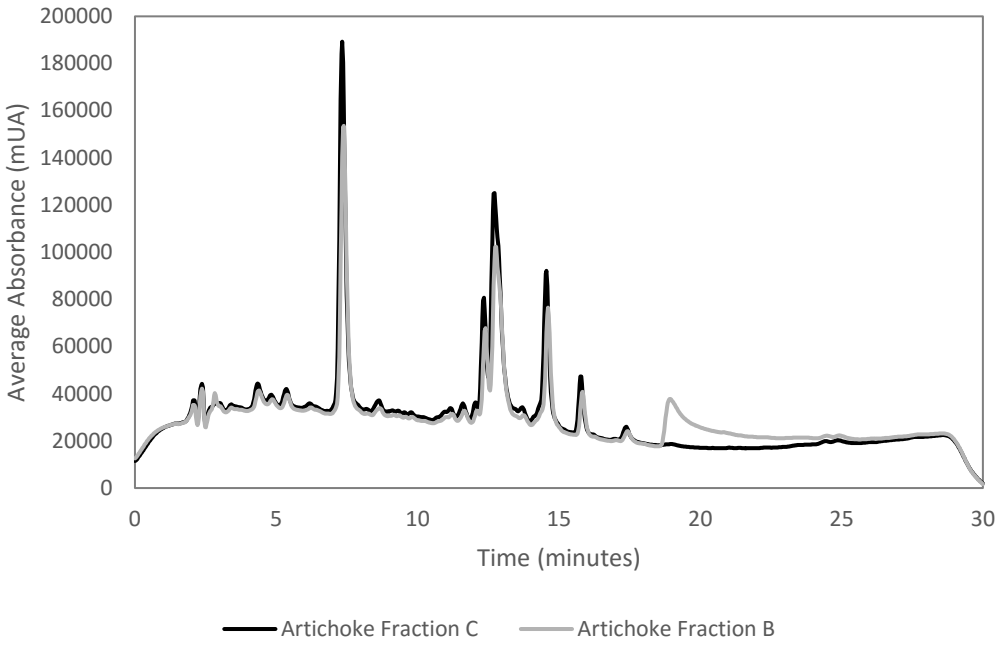


Figure 4.14 - HPLC-DAD analysis of the artichoke fractions B and C.

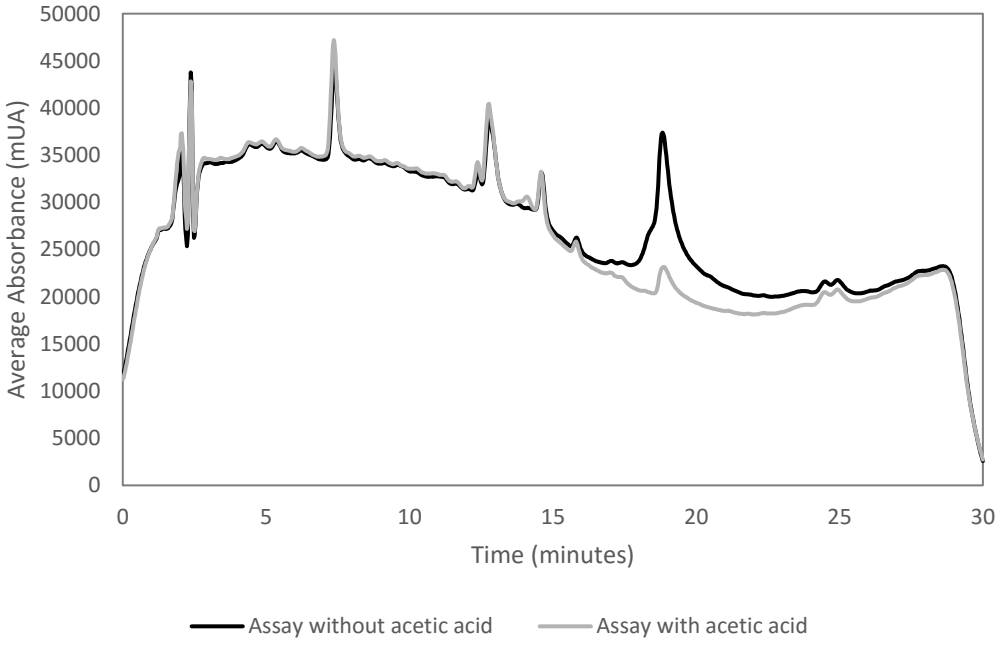


Figure 4.15 - HPLC-DAD analysis of the precipitates obtained from both methods, with and without acetic acid.

An analysis of variance (ANOVA) showed that the levels of phenols and tannins present in the precipitates and supernatants obtained from both assays didn't showed either

significant differences (p-value > 0.05) (Table 4.7), which means that the acetic acid doesn't prevent the precipitation of these compounds along with the polysaccharides and proteins present in the mucilage.

Table 4.7 - Quantification of total phenols and tannins in the supernatants and precipitates obtained with the mucilage precipitation methods with and without acetic acid. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **Concentration (µg/ mg of artichoke extract)** | | | |
|---|---|---|---|---|
| | Assay without acetic acid | | Assay with acetic acid | |
| | Artichoke Fraction C | Precipitate | Artichoke Fraction B | Precipitate |
| **Total Phenols** | 28 ± 3.5 | 4 ± 0.8 | 27 ± 2.1 | 4 ± 0.1 |
| **Taninns** | 1.8 ± 0.3 | 0.3 ± 0.04 | 2.0 ± 0.5 | 0.3 ± 0.1 |

In Table 4.8 is shown the quantification of chlorogenic acid, cynarin and cynaroside in the artichoke fractions B and C. Artichoke fraction C proved to be the richer fraction in chlorogenic acid, cynarin and cynaroside.

Table 4.8 - Quantification of chlorogenic acid, cynarin and cynaroside in the artichoke fractions B and C.

| | **Concentration (µg/mg of extract)** | |
|---|---|---|
| | Artichoke Fraction B | Artichoke Fraction C |
| Chlorogenic acid | 63 | 68 |
| Cynarin | 82 | 83 |
| Cynaroside | 14 | 18 |

The acetic acid in this method is commonly used to free the polysaccharides from the salts and to recover them by precipitation through the addition of ethanol. However, the results obtained from both chromatograms, from total phenols and tannins quantification and from the quantification of selected polyphenols, showed no differences between artichoke fractions B and C obtained from both mucilage precipitation methods, with and without acetic acid. Considering these results and taking into account the purpose of these compounds and their costs of production, the artichoke fraction C was the fraction that appeared to have more potential. Therefore, this fraction was studied more deeply.

In order to analyze the separation of the artichoke compounds by the mucilage precipitation method with no use of acetic acid, an analysis of the artichoke fraction C and the precipitate obtained from this procedure was performed by HPLC-DAD. In the chromatograms of Figure 4.16, the differences between the supernatant and the

precipitate obtained from this purification process are observed. Through a meticulous analysis, it's possible to spot the same peaks in both chromatograms, which means that artichoke extract compounds were dragged along with the mucilage, resulting in their precipitation too.

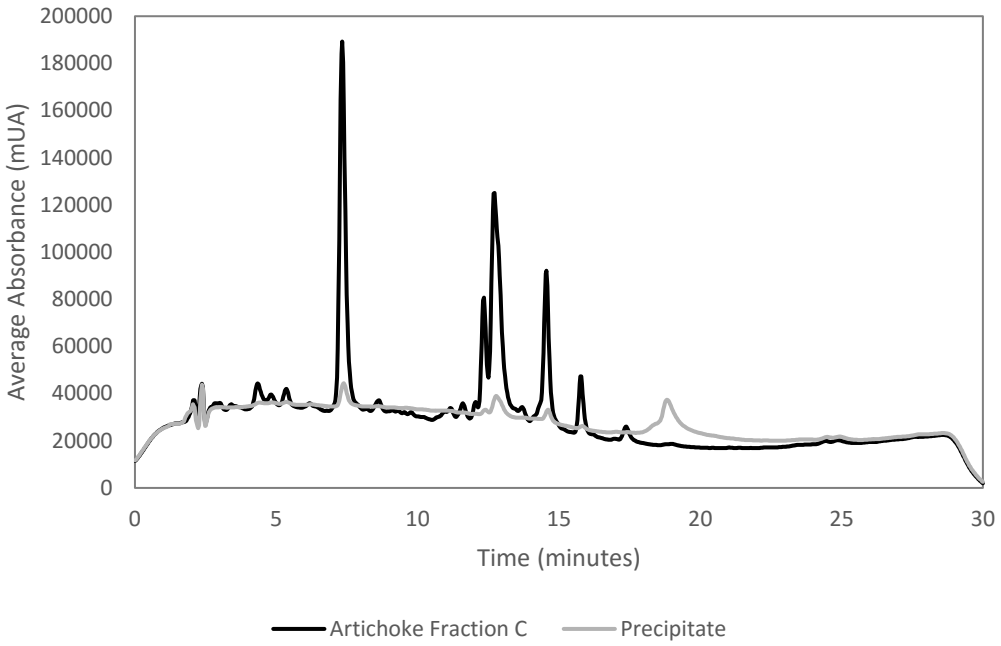


Figure 4.16 - Analysis of the artichoke fraction C and its precipitate by HPLC-DAD (1 mg/mL both).

In Table 4.9 is shown the quantification of chlorogenic acid, cynarin and cynaroside in the artichoke fraction C and its precipitate. Artichoke fraction C retained most of the selected polyphenols during the mucilage precipitation (68 µg/mg of extract of chlorogenic acid; 83 µg/mg of extract of cynarin; and 18 µg/mg of extract of cynaroside), while its precipitate dragged along a small fraction of those polyphenols during precipitation (9 µg/mg of extract of chlorogenic acid; 16 µg/mg of extract of cynarin; and 0.7 µg/mg of extract of cynaroside). The polyphenols precipitation may be due to the interactions between polyphenols and polysaccharides, which bond them forcing the polyphenols to precipitate along with the polysaccharides [17].

Table 4.9 - Quantification of chlorogenic acid, cynarin and cynaroside in the artichoke fraction C and its precipitate.

| | **Concentration (µg/mg of extract)** | |
|---|---|---|
| | Artichoke Fraction C | Precipitate |
| Chlorogenic acid | 68 | 9 |
| Cynarin | 83 | 16 |
| Cynaroside | 18 | 0.7 |

The boiling point of ethanol is 78ºC [18], which is lower than the water boiling point (100ºC), so a strategy to eliminate the ethanol present in the supernatant through a washing process by a rotary evaporator was adopted, and a HPLC-DAD analysis of washed and unwashed artichoke fractions C was made to assure that this process wouldn't change their composition, being the results in Figure 4.17. The chromatograms of both fractions are identical, having only different peaks intensities. Thus, the washing process didn't affect the compounds present in the supernatants, being safe to use.

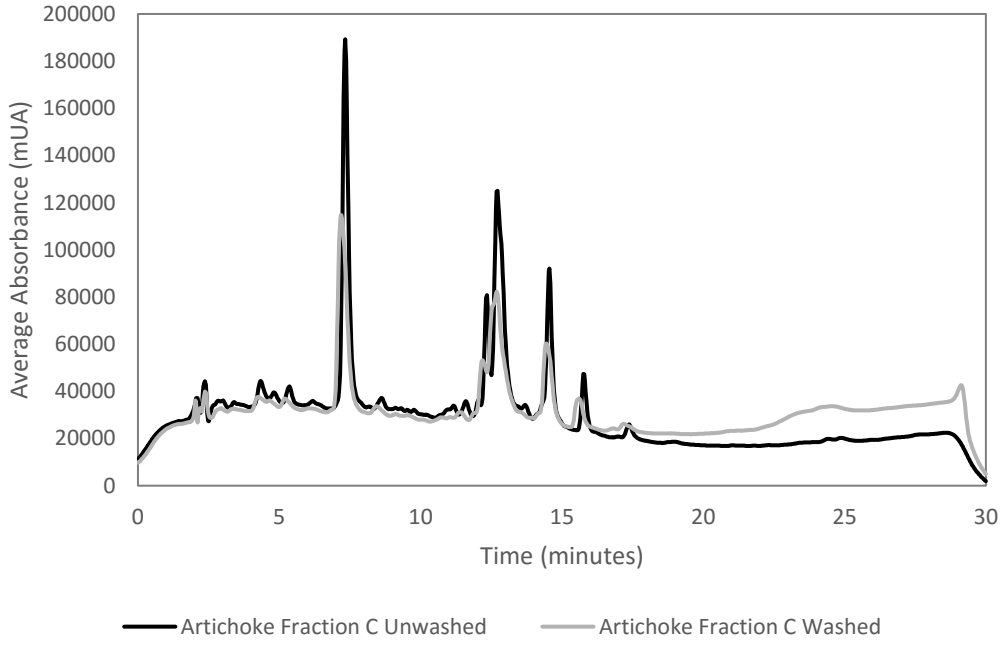


Figure 4.17 - HPLC-DAD analysis of the artichoke fraction C washed and unwashed.

A quantification of the total phenols present in the artichoke fraction C before and after this process was also done. As showed in Table 4.10, there was a loss of total phenols due to this process, but according to an analysis of variance (ANOVA), the difference isn't significant (p-value > 0.05). Thus, the washed artichoke fraction C will be used in the following studies.

Table 4.10 - Total phenols present in the artichoke fraction C before and after the wahing process. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **Concentration (µg/mg of artichoke extract)** | |
|---|---|---|
| | Artichoke Fraction C Washed | Artichoke Fraction C Unwashed |
| **Total Phenols** | 22.7 ± 2.4 | 28.3 ± 3.5 |

## 4.4. Antioxidant Activities: DPPH Assays

### 4.4.1. Artichoke Extract

The effect of the artichoke extract in terms of the DPPH radical scavenging activity is represented in Figure 4.18. During the assay, the colors alterations between samples with different artichoke infusion concentrations were visible, implying the existence of antioxidant activity. The artichoke extract showed a clear dose-response scavenging effect on the free radical DPPH with a singular pattern. The antioxidant activity of artichoke extract obtained through DPPH method, expressed as the half maximal effective concentration ($EC_{50}$), was 99 ± 29 µg/mL. Falé *et al*. [6] obtained an $EC_{50}$ of 123.1 ± 5.7, which is higher than the result obtained in this study.

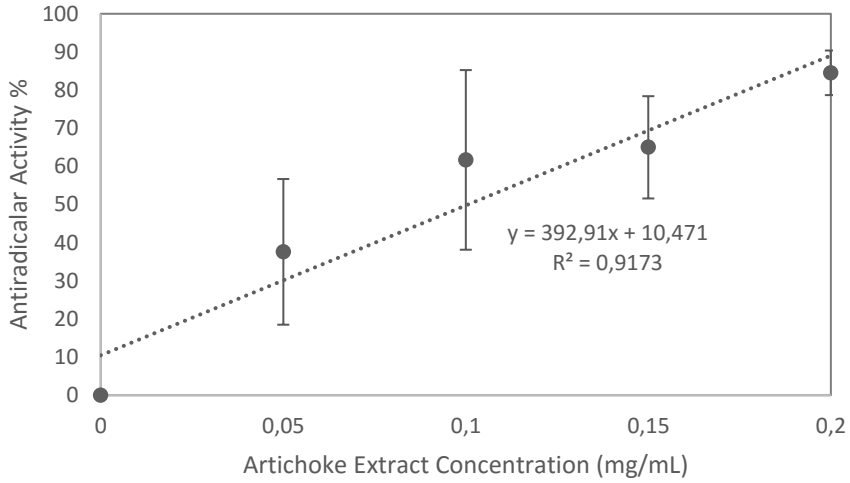


Figure 4.18 - Antioxidant activity of artichoke extract. The data are presented as the mean ± SD of at least 3 replicates experiments.

### 4.4.2. Artichoke Fraction A

Artichoke Fraction A showed a clear dose-response scavenging effect on the free radical DPPH, indicating an antioxidant capacity, being the results in Figure 4.19. The antioxidant activity, expressed as the quantity of extract necessary to decrease the initial DPPH radical concentration by 50% ($EC_{50}$), obtained for the artichoke fraction A was 95 ± 1.5 µg/mL. Due to the percentage stabilization, the value of antioxidant activity was taken directly from the graphic instead of the trendline equation.

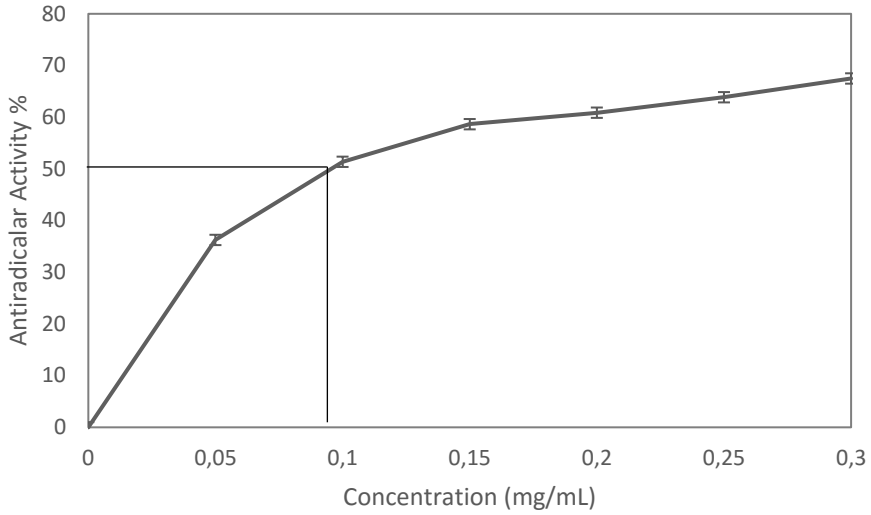


Figure 4.19 - Antioxidant activity pattern of Artichoke Fraction A abtained from dialysis. The data are presented as the mean ± SD of at least 3 replicates experiments.

## 4.4.3. Artichoke Fractions B and C

In Table 4.11 are the results for the artichoke fractions B and C antioxidant activities, and as it shows, the purify fraction with the best antioxidant activity was the artichoke fraction C (56 ± 6 µg/ mL) (Figure 4.20), while the artichoke fraction B had a poorer antiradicalar activity (67 ± 6 µg/ mL) (*Figure 4.21*).

Table 4.11 - Half maximal effective concentration ($EC_{50}$) of the artichoke fractions B and C obtained from the assays with and without acetic acid. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | Artichoke Fraction C | Artichoke Fraction B |
|---|---|---|
| **$EC_{50}$ (µg/ mL)** | 56.1 ± 6.05 | 66.5 ± 5.90 |

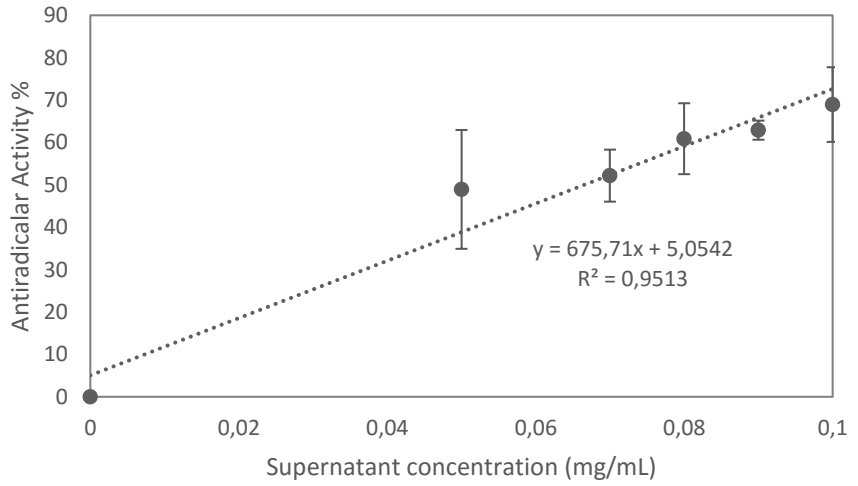


Figure 4.20 - Antiradicalar activity (%) of the artichoke fraction C. The data are presented as the mean ± SD of at least 3 replicates experiments.

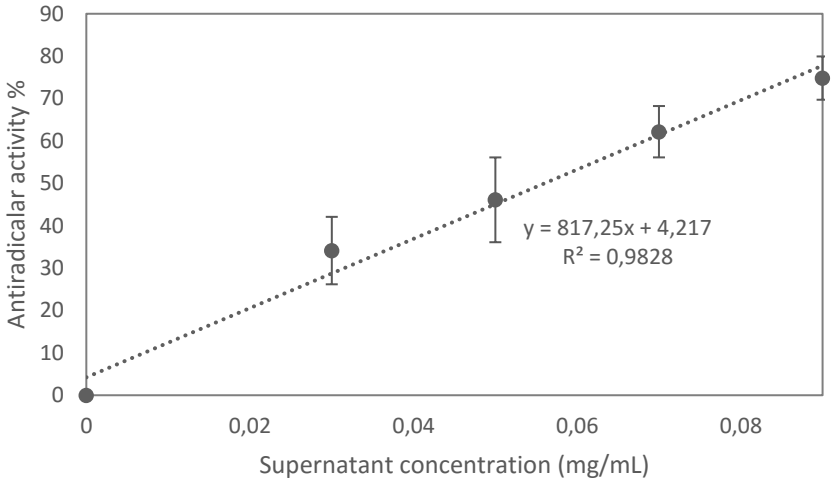


Figure 4.21 - Antiradicalar activity (%) of the artichoke fraction B. The data are presented as the mean ± SD of at least 3 replicates experiments.

### 4.4.4. Ascorbic Acid

Ascorbic acid was chosen as the antioxidant reference for this test, so it was needed to perform a DPPH assay with this antiradicalar compound. In Figure 4.22 is represented the antioxidant activity of the ascorbic acid, which $EC_{50}$ obtained was 2.8 ± 0,1 µg/ mL, which is similar to the results of other studies [21], [22].

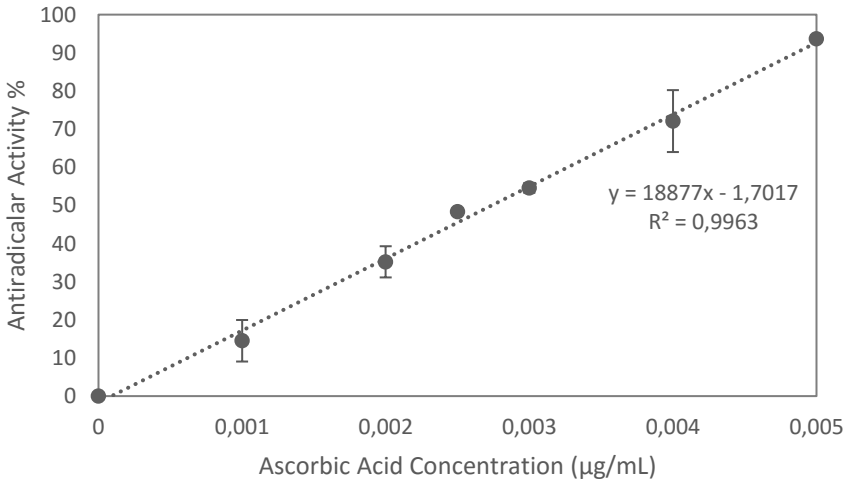


Figure 4.22 - Antioxidant activity of ascorbic acid. The data are presented as the mean ± SD of at least 3 replicates experiments.

The antioxidant activity of ascorbic acid is the highest when compared with the results of the artichoke extract and the fractions obtained from purification methods (Figure 4.23). However, the ascorbic acid is a pure compound, while the artichoke extract and the fractions are complex mixtures of several components.

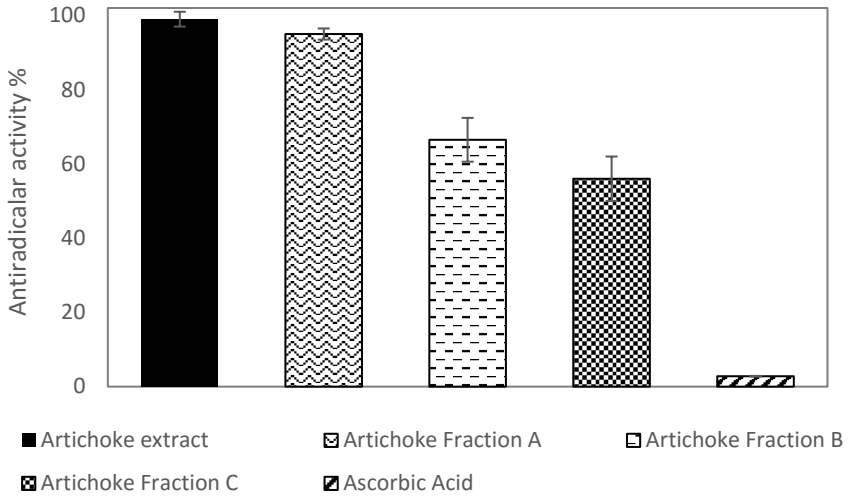


Figure 4.23 - Antioxidant activities of artichoke extract, artichoke fractions A, B and C, and ascorbic acid The data are presented as the mean ± SD of at least 3 replicates experiments.

## 4.5. Discussion

Overall, both methods tested to purify the artichoke extract in order to potentiate its capacities, dialysis and mucilage precipitation method without the use of acetic acid, were successful, being the results in Table 4.12.

Table 4.12 - Total content of chlorogenic acid, cynarin and cynaroside in the artichoke extract, artichoke fraction A obtained from dialysis and artichoke fraction C obtained from the mucilage precipitation method with no use of acetic acid, and their antioxidant activity. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | Artichoke extract | Artichoke Fraction A | Artichoke Fraction B | Artichoke Fraction C |
|---|---|---|---|---|
| **Total Phenols (µg/mg of extract)** | 31 ± 1 | 35 ± 10 | 27 ± 2.1 | 23 ± 2.4 |
| **Chlorogenic Acid (µg/mg of extract)** | 56 | 42 | 63 | 68 |
| **Cynarin (µg/mg of extract)** | 83 | 71 | 82 | 83 |
| **Cynaroside (µg/mg of extract)** | 7 | 7 | 14 | 18 |
| **Antioxidant Activity (µg/mL)** | 99 ± 2 | 95 ± 1,5 | 67 ± 6 | 56 ± 6 |

The polyphenolic content of artichoke extract increased after the acidic digestion and dialysis (31 ± 1 to 35 ± 10 µg/mg of extract), and decreased after the mucilage precipitation (31 ± 1 to 27 ± 2.1 and 23 ± 2.4 µg/mg in the case of the assays using and not using acetic acid, respectively). After dialysis, was observed a decrease of chlorogenic acid and cynarin after acidic digestion and dialysis, from 56 to 42 µg/mg of extract and from 83 to 71 µg/mg of extract respectively, while cynaroside concentration was maintained (7 µg/mg of extract). After mucilage precipitation, almost all compounds suffered an increase in the artichoke fractions B and C comparing with their original content in artichoke extract (from 56 to 63 and 68 µg/mg of extract in chlorogenic acid; from 83 to 82 and 83 µg/mg of extract in cynarin; and from 7 to 14 and 18 µg/mg in extract of cynaroside). In the three cases, the antioxidant activity improved from 99 µg/mL (artichoke extract) to 95 µg/mL in the artichoke fraction A resulted from dialysis, and to 67 and 56 µg/mL in the artichoke fractions B and C, respectively, obtained from the mucilage precipitation method.

Observing the results of Table 4.12, the mucilage precipitation method with no use of acetic acid appears to be more effective in improving the artichoke extract antioxidant activity. It was able to increase the antioxidant capacity over 40%. Therefore, the artichoke fraction C was the purified fraction chosen to continue the study and to be incorporated in the topical formulations.

## 4.6. Extracts Cytotoxicity

Cell-based assays are used to determine if a compound has effect on cell proliferation or direct effects that lead to cell death. In order to study the artichoke extract and the artichoke fraction C cytotoxicity, the MTT assay was performed using HaCaT cells, a spontaneously immortalize human keratinocyte cell line. In the MTT assay, the viable cells with active metabolism are capable of converting the MTT into a purple compound, the formazan, while the dead cells are incapable of converting it, which allows the quantification of viable cells [19]. The results of this assay are shown in Figure 4.24.

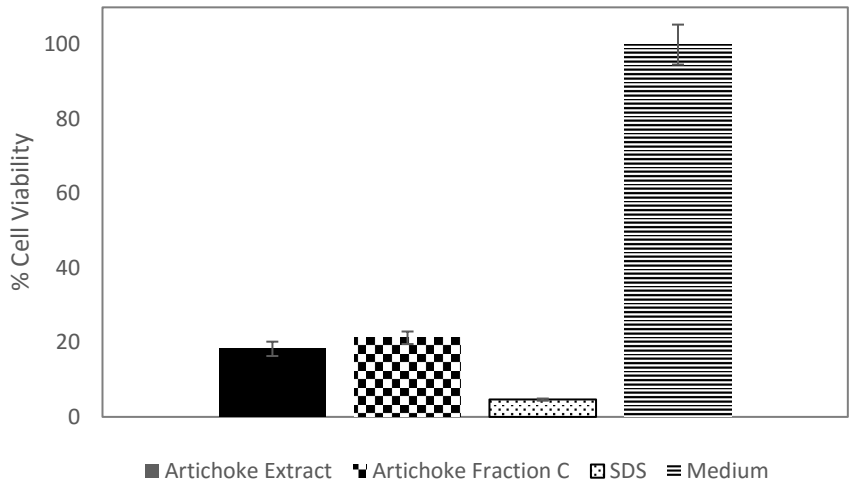


Figure 4.24 - Cytotoxicity of artichoke extract (black bar) and artichoke fraction C (square bar) (1 mg/mL). SDS (sodium dodecyl sulfate) (dots bar)) was used as positive control and the medium as negative control (horizontal risks bar). The data are presented as the mean ± SD of 6 replicates experiments.

Artichoke extract and artichoke fraction C showed a cell viability of 18.3% and 21.3% respectively, while SDS showed 4.6% and the medium 100%. Both fractions showed cytotoxicity and significant differences (p-value < 0.05) according to an analysis of variance (ANOVA), when compared with the medium (negative control) and the SDS (positive control). However, the HaCaT cell lines are more sensible than actually skin, since skin has a functional barrier that prevents the total absorption of these compounds when applied to skin. The rate-limiting barrier to the absorption of topical cosmetics is the stratum corneum, due to its length, which comprehends the number of cell layers, the thickness, the cell size and the difficulty of this pathway that the substances have to cross [20].

The $EC_{50}$ of each fraction was also determined, being the results in Table 4.13. The artichoke extract and the artichoke fraction C showed a cytotoxicity of 173 ± 1 mg/mL and 218 ± 1 mg/mL, respectively. Meaning that the artichoke fraction C is the fraction less toxic when in contact with HaCaT cells.

Table 4.13 - Cytotoxicity of the artichoke extract and artichoke fraction C. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **$EC_{50}$ (µg/mL)** |
|---|---|
| Artichoke Extract | 173 ± 1 |
| Artichoke Fraction C | 218 ± 1 |

## 4.7. Antioxidant Activities: Reactive Oxygen Species (ROS) scavenging activity

The anti-radicalar activity of the artichoke extract and the artichoke fraction C was tested in HaCaT cells exposed to $H_2O_2$. As a preliminary assay, it was tested whether the order of application in cells, samples 30 minutes before $H_2O_2$ or simultaneously, affected the response of ROS production by the cells. As showed in Figure 4.25, the application order doesn't affect the production of ROS by the cells, once there are no significant differences between the same samples applied before or simultaneously with the hydrogen peroxide. Therefore, it was decided to apply the fractions and the hydrogen peroxide simultaneously.

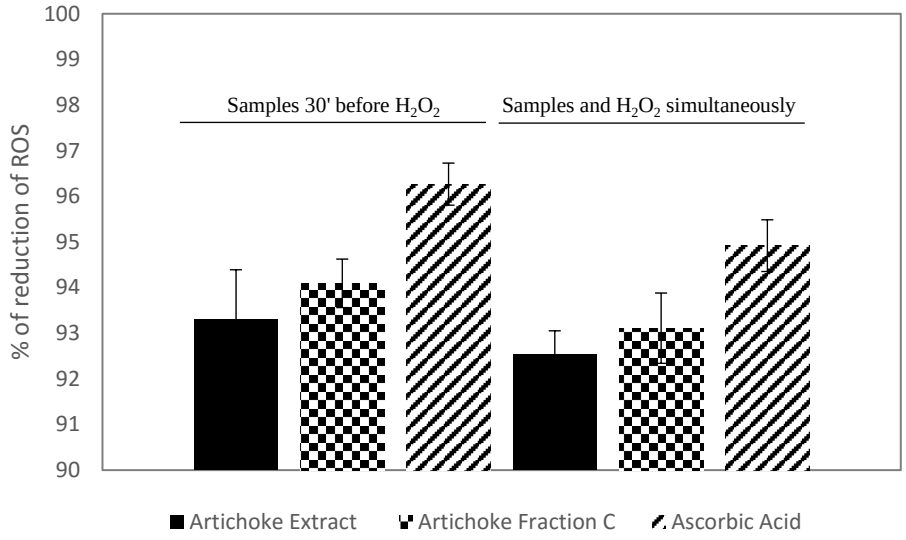


Figure 4.25 - ROS production after artichoke extract (black bars) and artichoke fraction C (square bars) (1 mg/mL) exposure to HaCaT cells in RPMI medium for 30 minutes before the addiction of $H_2O_2$ and at the same time. Ascorbic acid as a negative control (risks bars). The data are presented as the mean ± SD of at least 5 replicates experiments.

In order to study the ROS scavenging effect on the artichoke extract and the artichoke fraction C, the ascorbic acid was once again chosen as the antioxidant reference. As showed in Figure 4.26, both fractions revealed ROS scavenging activity when exposed to $H_2O_2$, a chemical compound responsible for inducing oxidative stress, at concentration of 1 mg/mL. In the assays were the $H_2O_2$ was placed 30 minutes before the samples, artichoke extract, artichoke fraction C and ascorbic acid showed a 93.3, 94.1 and 96.3 % of reduction of ROS, respectively. When the $H_2O_2$ was applied simultaneously with the samples, the ascorbic acid showed again the highest percentage of reduction of ROS with 94.9%, and the artichoke extract and the artichoke fraction C showed 92.5 and 93.1%, respectively. Analysis of variance (ANOVA) was performed to compare the two fractions

with the ascorbic acid, and the results showed significant differences in both cases (p-value < 0.05). This analysis also showed significant differences between both fractions (p-value < 0.05).

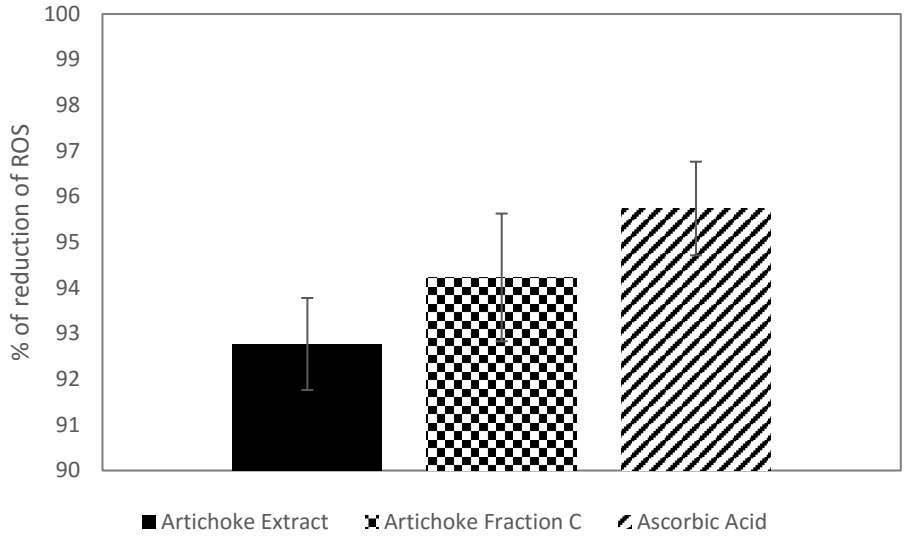


Figure 4.26 - ROS production of artichoke (black bar) and artichoke fraction C (squares bar) (1 mg/mL) in HaCaT cells in RPMI médium in presence of $H_2O_2$. Ascorbic acid was used as a negative control (risks bar). The data are presented as the mean ± SD of at least 10 replicates experiments.

Another assay was performed, in this case was used UVB radiation, a physical inducer of antioxidant stress, being the results in Figure 4.27. The artichoke extract and the artichoke fraction C showed 92.8 and 94.2% of reduction of ROS, while ascorbic acid showed 95.7%. In this case, the analysis of variance (ANOVA) didn't revealed any significant difference (p-value > 0.05) between the fractions and the ascorbic acid, and between both fractions.

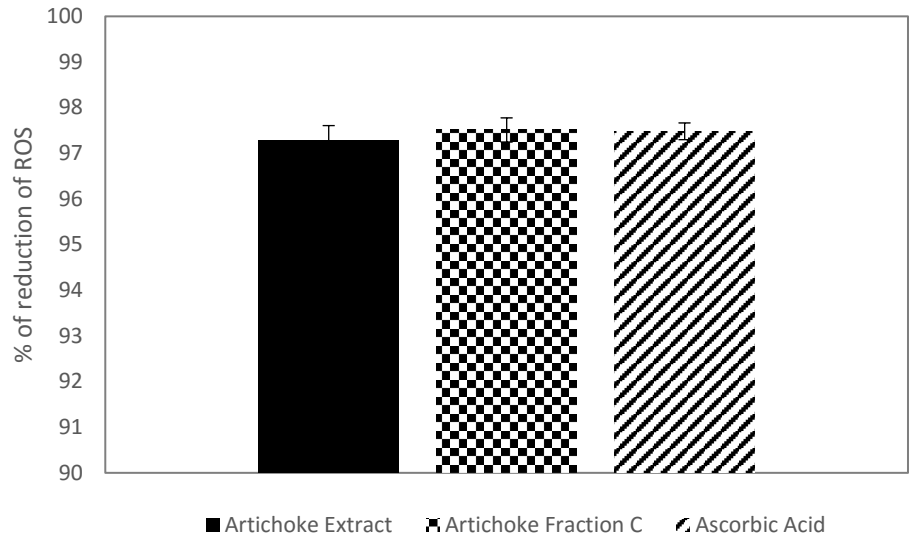


Figure 4.27 - ROS production of artichoke extract (black bar) and artichoke fraction C (squares bar) (1 mg/mL) in HaCaT cells in RPMI médium when the cells are exposed to UVB radiation for 15 minutes. Ascorbic acid (risks bar) was used as a negative control. The data are presented as the mean ± SD of at least 5 replicates experiments.

Taking into account the previous assays, the artichoke extract and the artichoke fraction C have powerful ROS scavenging effects, once their results are very similar with the ones obtained for the ascorbic acid, a well-known antioxidant. It should be noted that both fractions studied before, artichoke extract and artichoke fraction C, are complex mixtures composed of several chemical compounds while the ascorbic acid is a pure compound, which makes the results obtained in these assays even more remarkable.

## 4.8. Solar Protection Factor Assay

Flavonoids are plant pigments that are synthesized from phenylalanine, and several studies showed that these compounds have a wide spectrum of pharmacological properties, including antioxidative, antiallergic, anti-inflammatory, antidiabetic, hepato- and gastro-protective, antiviral, and antineoplastic activities, being extremely important to human health [23]. Therefore, the content of flavonoids was measure in the artichoke extract and the supernatant and the results are showed in Table 4.14.

Table 4.14 - Content of flavonoids in the artichoke extract and the artichoke fraction C. The data are presented as the mean ± SD of at least 3 replicates experiments.

| | **Flavonoids (µg/mg of extract)** |
|---|---|
| Artichoke Extract | 31 ± 2 |
| Artichoke Fraction C | 39 ± 7 |

Sunscreens are usually composed of synthetic chemical filters with a high capacity to absorb sun light at the region of UVB (320–290 nm) and UVA (400–320 nm) spectrum. Due to all the adverse effects (e.g. estrogenic effects, disruption of human endocrine activity, among others) of these compounds, there is a need to reduce the filter concentration in sunscreen formulations without affect their properties. A solution to this problem is to use natural products with a great antioxidant capacity (e.g. green coffee) instead of chemical filters, which may improve the photoprotective activity of sunscreen formulations. In fact, these natural products show several advantages, such as the bioactivity, relative safety and achievement from renewable sources, low cost, besides the feasibility for application in a wide range of health care products. As several studies suggest that one of the functions of the flavonoids is to protect the plants from potential harmful solar irradiation [24], [25], artichoke extract and the supernatant, rich sources of flavonoids and polyphenols, are potential candidates to replace the chemical filters in sunscreen formulation [26]. The SPF determination for the artichoke extract and the supernatant are represented on Table 4.15.

Table 4.15 - SPF found for the natural oils/extracts. The data are presented as the mean ± SD of at least 3 replicates experiments.

| Sample | SPF |
|---|---|
| Green coffee oil | 5.03 ± 0.23 |
| Artichoke Extract | 10.99 ± 0.29 |
| Artichoke Fraction C | 10.20 ± 0.21 |

The green coffee oil (GCO) was the chosen positive control, since there are several studies suggesting the use of GCO as a potential natural product for improving SPF in sunscreens formulations thus, allowing to decrease the concentration of chemical or physical filters in such formulations [27]–[29]. This natural oil showed a SPF of 5.03 ± 0.23, while the artichoke extract and the supernatant presented 10.99 ± 0.29 and 10.20 ± 0.21, respectively. An analysis of variance (ANOVA) was performed to compare both fractions with the GCO, and the results showed that there is a significant difference between them (p-values $< 0.05$), being the artichoke extract the compound with the highest SPF. Therefore, the artichoke extract and the supernatant appears to have an enormous potential to be applied in sunscreen formulations with the advantages that they are natural, harmless and powerful antioxidants, which prevents the ROS production due to solar radiation.

## 4.9. Topical Formulations Preparation

### 4.9.1. Final Formulations

The artichoke extract and the artichoke fraction C were chosen to be incorporated in topical formulations, as bioactive ingredients, and it was decided to develop two topical formulations for each fraction, a gel and a cream.

As the purpose of this study is to analyze the properties of the formulations containing the bioactive ingredients when applied in human skin, then it was decided to use the less ingredients possible to avoid raising doubts about the results obtained for the artichoke extract and the artichoke fraction C.

For both gels (containing the artichoke extract (AG) and the gel containing the artichoke fraction C (CG)), it was decided to use a well-known formulation with the only the basic ingredients. For the cream containing the artichoke extract (AC) and the cream containing the artichoke fraction C (CC), was decided to develop a new formula, and for that were chosen specific ingredients in accordance with the final formulation properties desired. The formulations details are fully described in Materials and Methods chapter.

Besides the formulations containing the bioactive ingredients, a blank gel (BG) and a blank cream (BC) were also prepared following the formulations chosen for each one. These blanks were used as a control for the following studies.

### 4.9.2. Physico-chemical characterization of formulations

#### 4.9.2.1. Rheology

Rheology characterization is very important in cosmetics once it helps reaching the optimal formulation that would be appealing to the consumer, choosing the production equipment and packaging, studying the formulation stability and it is considered a parameter in quality control [30], [31]. Therefore, in order to study the ability of the topical formulations to resist structural breakdown when applied to the skin, a shear-rate profile was traced for each formulation prepared. The representative flow curves are shown in Figure 4.28 and Figure 4.29. Apparent viscosity decreases concurrently with

the increase of shear rate, meaning that all formulations prepared are considered shearthinning.

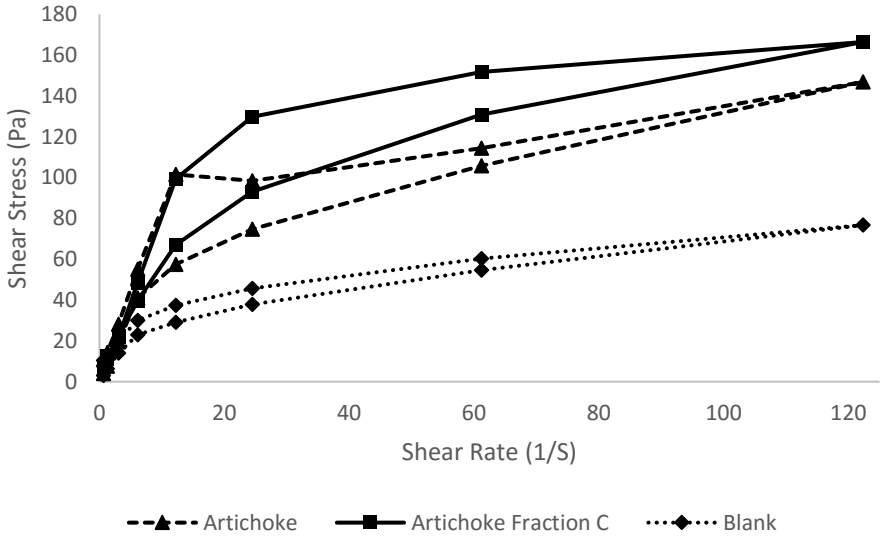


Figure 4.28 - Flow curves of artichoke extract, artichoke fraction C and blank creams representing shear stress as function of shear rate.

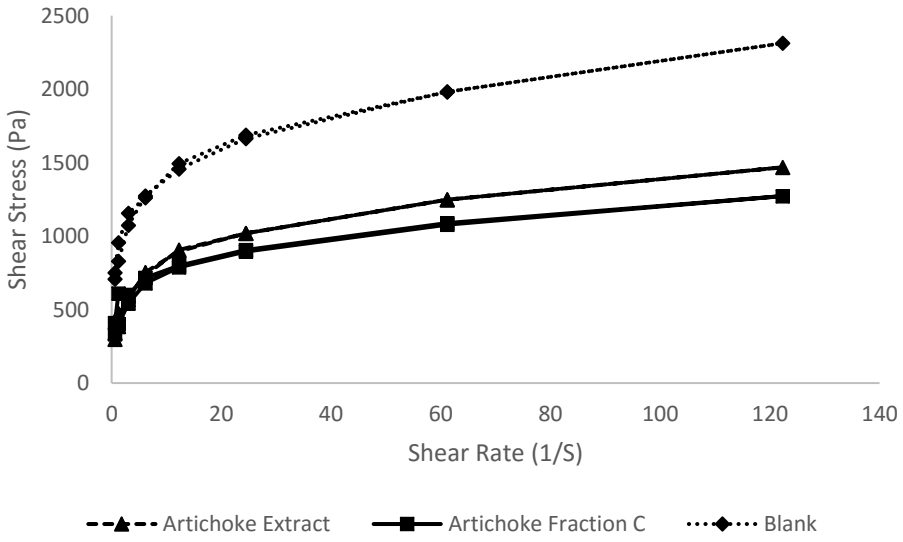


Figure 4.29 - Flow curves of artichoke extract, artichoke fraction C and blank gels representing shear stress as function of shear rate.

In the case of the creams, the flow curves also exhibited hysteresis loops, meaning that the formulation suffers a structural breakdown when the stress is applied, and consequently the viscosity decreases, but the network is regenerated by energy interactions when the stress applied diminishes, and the original viscosity is restored. This behavior is typical of time-dependent fluids, since after a sufficient resting time, the

original viscosity it recuperated. The viscosity values presented in Table 4.16 show that the artichoke fraction C cream is the most and the blank cream is the less viscous formulation. The polysaccharides present in the plant extracts are possible responsible for the rheology behavior observed, since these swell in contact with water [32]. Therefore, the emulsions with artichoke extract or artichoke fraction C incorporated showed high viscosity due to the polysaccharides swelled, while the blank showed the lowest viscous behavior because it didn't have these compounds present in it. The results obtained are in accord once the artichoke fraction C is the richer fraction in polyphenols, and consequently, the most viscous formulation. The opposite was observed for the gel formulations, since the artichoke fraction C gel was the formulation that presented the lower and the blank the highest viscosity, as represented in Table 4.16. This behavior may be due to the different pH observed before the addition of sodium ions and to the sodium ions themselves, once pH and ionic strength are both parameters that can influence the rheology properties of carbopol gels [33].

Table 4.16 - Apparent viscosity values calculated at 61,18$s^{-1}$ of all formulations.

| Formulations | Apparent Viscosity (Pa.s) at 68,18 $s^{-1}$ |
|---|---|
| Artichoke Fraction C Cream | 2.48 |
| Artichoke Cream | 1.9 |
| Blank Cream | 0.9 |
| Artichoke Fraction C Gel | 17.8 |
| Artichoke Gel | 20.4 |
| Blank Gel | 32.4 |

#### 4.9.2.2. pH Values

The pH value of the topical products is a very important parameter, since the pH value can influence not only the solubility and stability of the bioactive ingredient in the formulations but also its release from the vehicle and its penetration into the skin barrier [34], [35]. Therefore, it's important to evaluate this parameter in each formulation produced. If it was out of the ideal pH range, it was corrected adding HCl (decrease pH value) or NaOH (increase pH value), depending of the pH value measured. The pH values of all formulations prepared are represented in Table 4.17 and all were between 5.4 and 6.4, meaning that all fit well within the ideal pH range for the skin.

Table 4.17 - Determination of pH values of all formulations prepared at environment temperature. The data are presented as the mean ± SD of at least 3 replicates experiments.

| Formulations | pH value |
|---|---|
| Artichoke Cream | 5.9 ± 0.4 |
| Artichoke Fraction C Cream | 5.9 ± 0.8 |
| Blank Cream | 6.4 ± 0.3 |
| Artichoke Gel | 5.4 ± 0.2 |
| Artichoke Fraction C Gel | 5.7 ± 0.4 |
| Blank Gel | 5.4 ± 0.2 |

### 4.9.3. Microbiological Control

Cosmetics have several ingredients in their constitution that are carbon sources, like water, oil and glycerin, and nitrogen sources, like amino acid derivatives and proteins, for fungi, bacteria and others microorganisms. Microbiological contamination of cosmetics products can lead to their instability, loss of safety and even the loss of their active ingredients. Therefore, it's necessary to use preservatives in cosmetics formulations in order to prevent the growth of bacteria or fungi. In the artichoke extract and supernatant formulations parabens, in particular methyl paraben and propyl paraben, were used. Besides that, the water activity (amount of water available for biological processes) in all formulations was reduced by adding glycerin, which has the capacity to bind with water, reducing this way the water available in the products, and consequently, naturally preserving the formulations from contamination [36].

Thus, a microbiological control is a regulatory requirement for cosmetics in order to ensure the microbial safety of the it, to maintain the its quality and specifications and to guarantee hygienic and high-quality handling [37].

Total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC) tests were performed through the spread-plate method for each formulation, and the results showed less than 10 colonies in both tests, which resulted from environmental contamination, meaning that the result obtain in all cases was less than 100 CFU per gram or milliliter of product tested. This value is lower than the limit imposed by the ISO 16212:2008 and ISO 21149:2006 defined for these products (Category 2), thus the products prepared are considered microbiologically safe.

### 4.9.4. Formulations Cytotoxicity

To test the cytotoxicity of all formulations prepared, gels and emulsions, the cell viability was evaluated using HaCaT cell lines in a MTT assay. The results obtained for the formulations containing artichoke and artichoke fraction C were compared to their blanks, as showed in Figure 4.30.

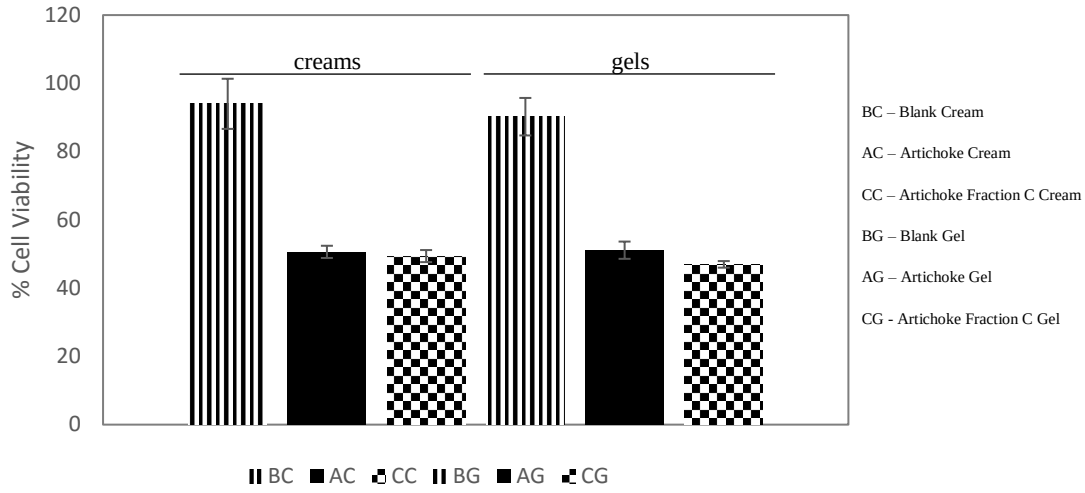


Figure 4.30 – MTT assay measurement of cytotoxicity of topical formulations containing the artichoke extract (black bars) and artichoke fraction C (squares bars) and their blanks (vertical risks bars). The data are presented as the mean ± SD of 6 replicates experiments.

The creams containing the artichoke extract and the artichoke fraction C showed 51% and 49% of cell viability, respectively. While the gel containing the artichoke extract showed 51% and the gel with the artichoke fraction C incorporated showed 47%. The cream blank showed 94% and the gel blank 90% of cell viability. Analysis of variance (ANOVA) showed that the emulsions containing the fractions weren't significantly different between them ($p$-value > 0.05). However, a significant difference ($p$-value < 0.05) was noted between the emulsions containing the bioactive ingredients and the blank. The same results were obtained for the gels.

All the formulations containing bioactive ingredients had a poorer cell viability compared to its blanks, which aren't harmful to the cells taking into account each ingredient present. The formulations showed a cell viability around 50% when compared to its blanks. Therefore, these results show that AC, AG, CC and CG can preliminary be considered as safe.

When compared the cell viability of both extracts and their corresponding formulations, it is possible to observe a substancial increase of the cell viability from the isolated fractions to the formulations, being the results in Figure 4.31. Analysis of variance (ANOVA) showed significant differences (p-value < 0.05) between the cell viability of extracts and their respective formulations. Therefore, the vehicle (emulsion or cream) is able to reduce the cytotoxicity of artichoke extract and artichoke fraction C.

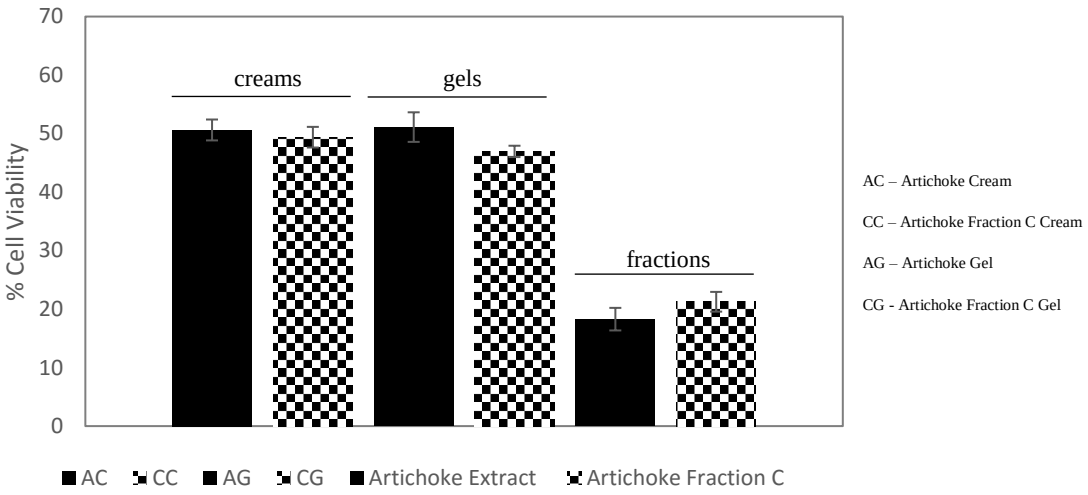


Figure 4.31 - MTT assay measurement of cytotoxicity of artichoke extract (black bars) and artichoke fraction C (square bars) and their respective topical formulations. The data are presented as the mean ± SD of 6 replicates experiments.

### 4.9.5. Effects on membrane integrity

In order to evaluate the effects of the formulations containing the bioactive ingredients on the membrane integrity of cells, an assay using propidium iodide was realized. This compound has the can bind to cellular DNA and as it is impermeant to the plasma membrane, it has the ability to only enter and stain damaged cells. The results are showed in Figure 4.32.

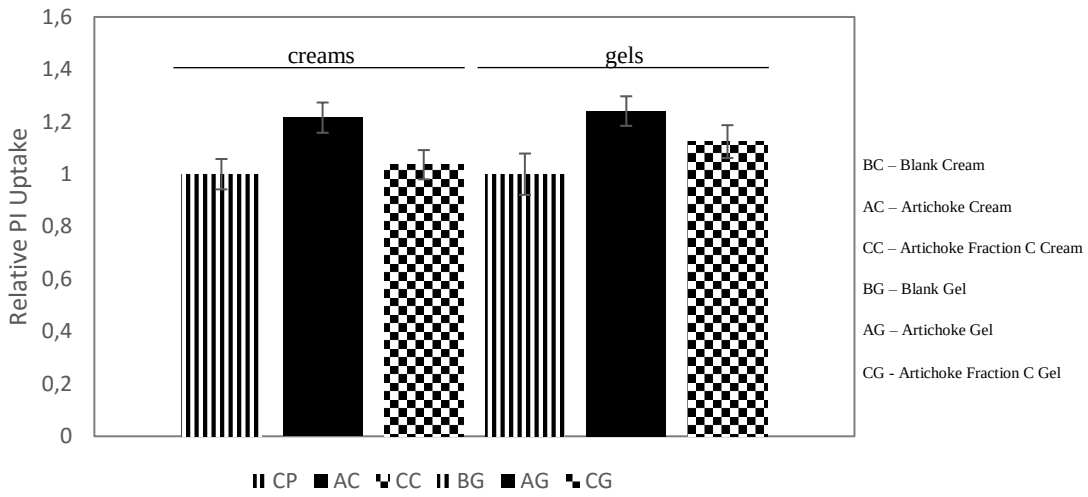


Figure 4.32 – Propidium iodide assay measurement of cytotoxicity of topical formulations containing the artichoke extract (black bars) and artichoke fraction C (squares bars) and their blanks (vertical risks bars). The data are presented as the mean ± SD of 6 replicates experiments.

Both blanks showed a relative propidium iodide (PI) uptake of 1. The gels with the artichoke extract and the artichoke fraction C incorporated showed a slightly higher relative PI uptake of 1.2 and 1.1, respectively. The creams containing the artichoke extract and artichoke fraction C showed a relative PI uptake of 1.2 and 1, respectively. Analysis of variance (ANOVA) showed that the cream containing the artichoke fraction C and the blank weren't significant different between them (p-value > 0.05). However, a significant difference (p-value < 0.05) was noted between the emulsion containing the artichoke fraction and the emulsion blank. The creams containing the bioactive ingredients were also significant different between them. The same analysis showed that the gels containing the bioactive ingredients were significant different between them and when compared with their blank.

Considering that the cell lines are a more sensitive system than the actual skin cells and that the differences between the AC and CC formulations and their blank isn't substantial, the results showed that all formulations can be considered not harmful.

## 4.10. Discussion

Reactive oxygen species (ROS) scavenging activity, solar protection factor (SPF) and cytotoxicity were studied for the artichoke extract and artichoke fraction C in order to compare both extracts. These in vitro studies showed that both fractions had powerful

ROS scavenging effects either in the presence of H2O2 or UVB radiation, and that both had a similar SPF value (10.99 for artichoke extract and 10.20 for artichoke fraction C). Cytotoxicity assays revealed also a similar cell viability for both fractions (18.3% for artichoke extract and 21.3% for artichoke fraction C). Since the results in all assays were identical for artichoke extract and artichoke fraction C, it was decided to incorporate both in topical formulations.

The topical formulations, a gel and a cream, containing the fractions were subjected to microbiological control, cytotoxicity and membrane integrity assays in order to evaluate their safety for human use. All formulations were considered safe in all assays realized.

Artichoke extract and artichoke fraction C and their respective formulations exhibited similar results in all assays performed. However, the costs and time of production of both fractions are very different. Artichoke fraction C required a larger amount of time and money to be produced, once it needs to be subjected to several procedures, including the precipitation of mucilage, and the washing and lyophilization processes. Therefore, taking into account the results and the costs and time of production of each fraction, it was decided to use only the formulations containing the artichoke extract in the following *in vitro* and *in vivo* studies.

## 4.11. *In Vitro* Studies: Reactive Oxygen Species (ROS) scavenging activity

In order to study the ROS scavenging effect of the gel and the cream containing the artichoke extract, the ascorbic acid was once again chosen as the antioxidant reference. As showed in Figure 4.33, both formulations revealed ROS scavenging activity when exposed to $H_2O_2$. The artichoke extract gel, artichoke extract cream and the ascorbic acid showed a 92.8, 90.3 and 95.7% of ROS reduction, respectively. Analysis of variance (ANOVA) was performed and the results showed significant differences ($p$-value $< 0.05$) between both formulations and between each formulation and the ascorbic acid.

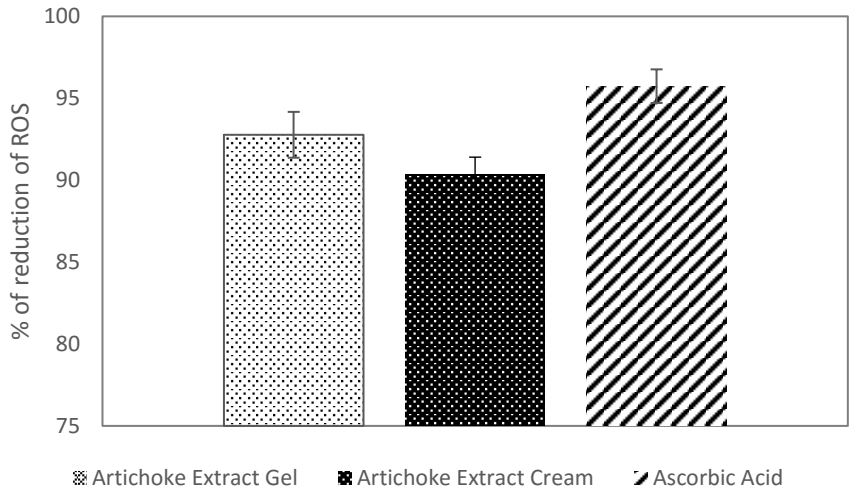


Figure 4.33 - ROS production of artichoke extract gel (white bar with black dots) and artichoke extract cream (black bar with white dots) in HaCaT cells in RPMI médium in presence of $H_2O_2$. Ascorbic acid was used as a negative control (risks bar). The data are presented as the mean ± SD of at least 5 replicates.

Another assay using UVB radiation as an inducer of antioxidant stress was performed, being the results in Figure 4.34. The artichoke extract gel, artichoke extract cream and the ascorbic acid showed an 80.5, 79.6 and 97.5 % of ROS reduction, respectively. Analysis of variance (ANOVA) was performed and the results showed significant differences (p-value < 0.05) between the both formulations and the ascorbic acid. However, no significant differences were observed between the gel and the cream containing the artichoke extract. In this assay, the gel containing the artichoke extracts revealed the highest antiradicalar activity.

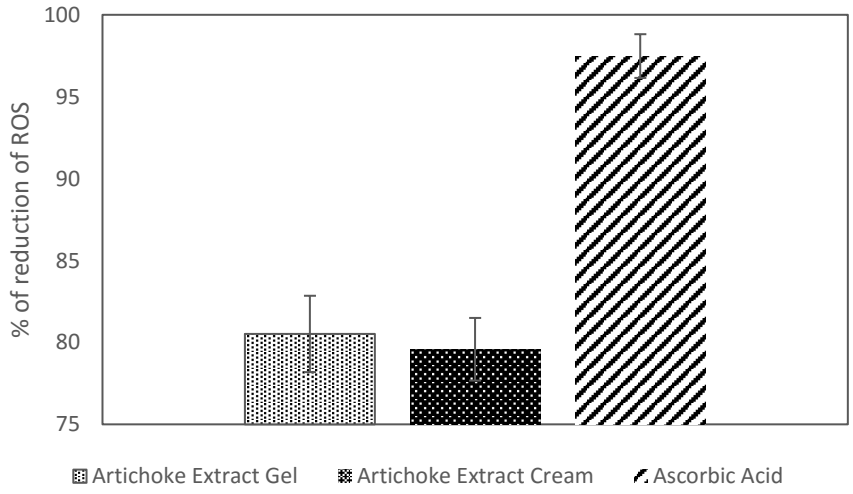


Figure 4.34 - ROS production of artichoke extract gel (white bar with black dots) and artichoke extract cream (black bar with white dots) in HaCaT cells in RPMI médium when the cells are exposed to UVB radiation for 15 minutes. Ascorbic acid was used as a negative control (risks bar). The data are presented as the mean ± SD of at least 5 replicates.

## 4.12. *In Vivo* Studies

### 4.12.1. Human Repeat Insult Patch Testing (HRIPT)

No evidence for the induction of allergic contact hypersensitivity was observed during the HRIPT assay, neither in the induction or in the challenge period. Therefore, the formulations showed very good skin compatibility and didn't exhibited allergenic potential, which makes them skin-friendly and dermatologically safe products.

### 4.12.2. Assessment of the protective effect against oxidative stress after UV radiation by chromameter evaluation

UV radiation is one of the factors that induce photo damage in the skin due to the production of reactive oxygen species (ROS) also known as free radicals. To prevent the reactions of free radicals, the human body has developed defense mechanisms known as antioxidants, however they can be inhibited by UV radiation and increase the formation of more ROS.

β-carotene is a Yellow chromophore molecule and when is oxidized by UV radiation it loses its chromophore capability and color. This discoloration can be monitored by colorimetry (b* parameter). Skin color is obtained using a tristimulus color analyzer that measures reflected color. The system uses β-carotene high sensitivity photocells filtered to match CIE standard. The measuring head contains a high-power xenon arc lamp which provides diffuse illumination from a controlled angle for vertical viewing and constant lighting on the object. The system detects any slight deviation in the xenon's light spectral distribution. The system provides data for the Luminance (L*), a* (red-green) and b*(blue-yellow) color distribution.

The protective effect against oxidative stress was evaluated by a Minolta Chromameter CR-400 (Minolta, Japan) in order to obtain the b* color, which is a measure along the yellow-blue axis (positive b* relates to the amount of yellowness and a negative b* to the amount of blueness), before and after the application of the products, including the formulations containing artichoke extract. β-carotene was the chromophore chosen as a reference in this procedure. The results obtained for the b* color measurement in the first day (D0) and the last day (D28) of the study are presented in Table 4.18.

Table 4.18 - Summary results of the evolution of b* color (AU) during the HRIPT assay.

| | | Time | n | Basal | Before irradiation | After irradiation |
|---|---|---|---|---|---|---|
| b* | Artichoke Gel + B-carotene | D0 | 10 | 16,8 | 36,3 | 33,2 |
| | | D28 | 10 | 16,8 | 35,8 | 34,3 |
| | Artichoke Cream + B-carotene | D0 | 10 | 16,9 | 41,6 | 38,6 |
| | | D28 | 10 | 16,9 | 40,0 | 38,1 |
| | Control | D0 | 10 | 14,8 | 40,0 | 38,2 |
| | | D28 | 10 | 15,0 | 36,6 | 36,0 |

To evaluate the true anti-oxidant activity obtained in each area at the end of the study, a relative analysis regarding D0 was performed, being the results summarized in Figure 4.35. A product shows antioxidant activity when a decrease in the oxidant activity of the UVA radiation occurs.

Regarding the anti-oxidant capacity, the product artichoke extract gel presented an 87.0% decrease in the oxidant activity of the UVA radiation. At the same time, the product artichoke extract cream presented an 75.8% decrease in the oxidant activity of the UVA radiation. Analysis of variance (ANOVA) showed significant difference (p-value < 0.05) between artichoke extract gel and the control, and the opposite occurred for the artichoke extract cream. Therefore, the artichoke extract gel presented a true *in vivo* antioxidant activity.

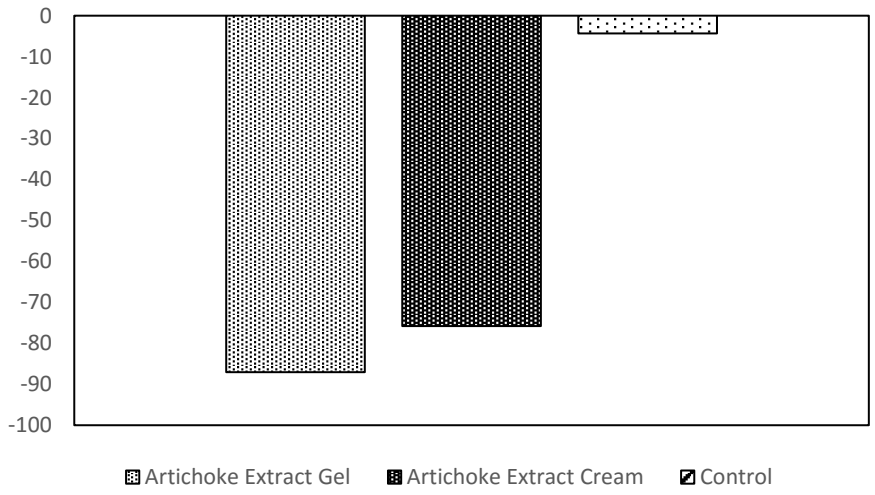


Figure 4.35 - Oxidant activity % change during the study after the exposure to artichoke extract gel (white bar with black dots) and artichoke extract cream (black bar with white dots). Mean values of all the subjects (n=10).

## 4.13. Discussion

In the ROS scavenging activity assays using $H_2O_2$ as inducer of antioxidant stress, the artichoke extract showed a ROS reduction of 92.5%, while the gel and the cream containing it showed 92.8 and 90.3%, respectively. In the assays using UV radiation, artichoke extract, and the gel and cream containing it presented 92.8, 80.5 and 79.6% of ROS reduction. Overall, the artichoke extract resulted in a higher percentage of ROS reduction. This result is probably due to the availability of the artichoke extract, free form or incorporated in a matrix. Aqueous artichoke extract when applied in the cells allows the direct contact of artichoke extract in its free form with the cells, resulting in an immediate antiradicalar action. On the opposite, the matrices of the formulations, cream and gel, are able to retain the artichoke extract, releasing it slowly to the medium with the cells, thus the action of it in has less impact due to a more extended response.

*In vivo* studies showed that the gel containing the artichoke extract presented an 87.0% decrease in the oxidant activity induced by UVA radiation, while the artichoke extract cream presented 75.8%. These results are in accordance with the ones obtained from the latest *in vitro* studies, since the gel with the artichoke extract incorporated showed the highest antioxidant capacity in both cases. Release profiles of the artichoke extract from both formulations can explain the differences of antioxidant activities observed between the cream and the gel. In this case, the matrix of the gel may be able to release the artichoke extract in a more efficient and faster way than the matrix of the cream. Patel *et al.* [38] studied the percentage of release of psoralen from two distinct formulations, a gel and a cream, and the results also showed a higher release profile of psoralen from the gel formulations than that from the cream formulations.

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# 5. Chapter: Conclusion

*Cynara Cardunculus* var. *Scolymus* is a rich source of polyphenols, which are responsible for its antioxidant properties, and it is highly produced by Mediterranean countries, such as France, Spain, Italy and Greece. Currently, plant extracts are being preferable to be used in cosmetics products due to the prohibition of the use of animal origin ingredients to an increase search for ecofriendly and sustainable products. Therefore, artichoke can potential be a specie of interest to be incorporated in cosmetic products with antioxidant purposes.

The aim of this study was to investigate the antioxidant properties of artichoke extract and its use as a bioactive ingredient in topical formulations with antioxidant purposes.

Infusion revealed to be an efficient procedure to extract bioactive compounds from artichoke bracts. Regarding polyphenols, an analysis of artichoke extract revealed the presence up to 56 of chlorogenic acid, 7 of cynaroside and 83 of cynarin, which values were expressed in μg/mg of artichoke extract. A characterization of artichoke extract showed that it is composed by 62.8% of fibers, 3.06 of polyphenols, 0.82% of carbohydrates and 0.002% of proteins. DPPH radical scavenging activity assay of artichoke extract was realized and it was obtained an $EC_{50}$ of 99 μg/mL.

Purification of artichoke extract through an acidic digestion with gastric juice followed by a 24 hours' dialysis, which resulted in artichoke fraction A, proved to be efficient in improving polyphenols content, from 31 to 35 μg/mg of artichoke extract. However, the amount of extractives, expressed in μg/mg artichoke extract, decreased from 56 to 42 for chlorogenic acid, from 83 to 71 for cynarin and for cynaroside the value remained equal. Concerning its antioxidant activity, was recorded an improvement from 99 to 95 μg/mL.

Mucilage precipitation method was tested using two different approaches, one using acetic acid and the other only using ethanol, which resulted in artichoke fractions B and C, respectively. Regarding polyphenols, artichoke fraction B exhibited 27 μg/ mg of artichoke extract and artichoke fraction C exhibited 23 μg/ mg of artichoke extract. DPPH assay revealed an $EC_{50}$ of 67 and 56 to artichoke fractions B and C, respectively. A chemical analysis of artichoke fraction B revealed the presence of 63, 82 and 14 μg/ mg

of artichoke extract of chlorogenic acid, cynarin and cynaroside, respectively; artichoke fraction C revealed the presence of 68, 83 and 18 μg/ mg of artichoke extract of chlorogenic acid, cynarin and cynaroside, respectively.

An overview of the results obtained from purification methods showed that artichoke fraction C was the fraction with better antioxidant activity. However, this was also the fraction with lowest phenolic content, despite the increased content of chlorogenic acid, cynarin and cynaroside. But since the main goal was to incorporate the fraction with better antioxidant properties in topical formulations with antioxidant purposes, the artichoke fraction C was the chosen one to proceed with the study.

Cytotoxicity assays in HaCaT cell lines revealed that both fractions decreased cell viability to 18.3% in artichoke extract and 21.3% in artichoke fraction C. ROS scavenging activity assay using hydrogen peroxide and UVB radiation revealed powerful antioxidant activity for artichoke fraction C and artichoke extract. In the assay using $H_2O_2$, ascorbic acid reduced 94.9% of ROS, while artichoke extract and artichoke fraction C reduced 92.5 and 93.1%, respectively. In the UVB radiation assay, significant differences (p-value $< 0.05$) between ascorbic acid, artichoke extract and artichoke fraction C weren't observed; artichoke extract and the artichoke fraction C showed 92.8 and 94.2% of ROS reduction, while ascorbic acid showed 95.7%. Both fractions also revealed to be rich in flavonoids (31 μg/mg of extract for artichoke extract and 39 μg/mg of extract for artichoke fraction C) and to have solar protection factor, which values were found between 10 and 11.

Topical formulations, a cream and a hydrogel, were prepared and both fractions, artichoke extract and artichoke fraction C, were incorporated in them. A physical-chemical characterization was performed for each formulation and all products showed acid pH values and shearthinning behavior, thus they were considered suitable for skin application. Assays of cytotoxicity and effects on membrane integrity in keranocytes, and a microbiological control showed that all formulations were also suitable for topical application.

Observing the results obtained in the analysis of both fractions and their respective formulations, and taking into account the costs and time of production of each fraction, formulations containing the artichoke extract, a cream and a hydrogel, were chosen to be

used in more *in vitro* studies and *in vivo* studies. *In vitro* studies included a ROS scavenging activity assay using hydrogen peroxide and UVB radiation, and *in vivo* studies included a Human Repeat Insult Patch Testing (HRIPT) and an assessment of the protective effect against oxidative stress after UV radiation by chromameter evaluation.

In the *in vitro* studies using $H_2O_2$ as oxidative stress inducer, the gel and the cream containing the artichoke extract showed a ROS reduction of 92.8 and 90.3%, respectively. In the assay with UVB radiation, the gel showed 80.5% and the cream showed 79.6% of ROS reduction. Overall, the formulations presented a lowest antioxidant activity against both inducers when compared with the artichoke extract in its free form (92.5% for $H_2O_2$ assay; 92.8% for the UV radiation assay).

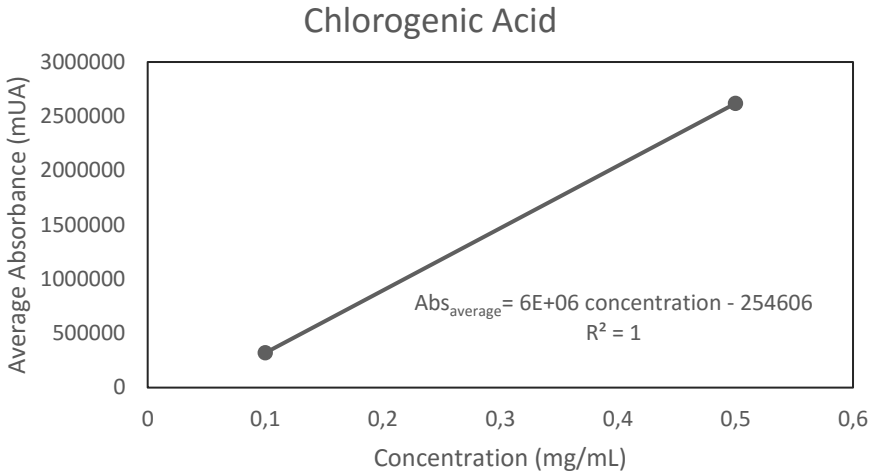
*In vivo* studies performed in volunteers demonstrated very good skin compatibility and no allergenic potential for both formulations, so they were considered dermatologically safe. Regarding antioxidant capacity, artichoke gel and cream exhibited an 87.0% and 75.8% decrease in the oxidant activity of the UVA radiation, respectively. In this assay, the artichoke gel was the topical formulation that presented a true *in vivo* antioxidant activity.

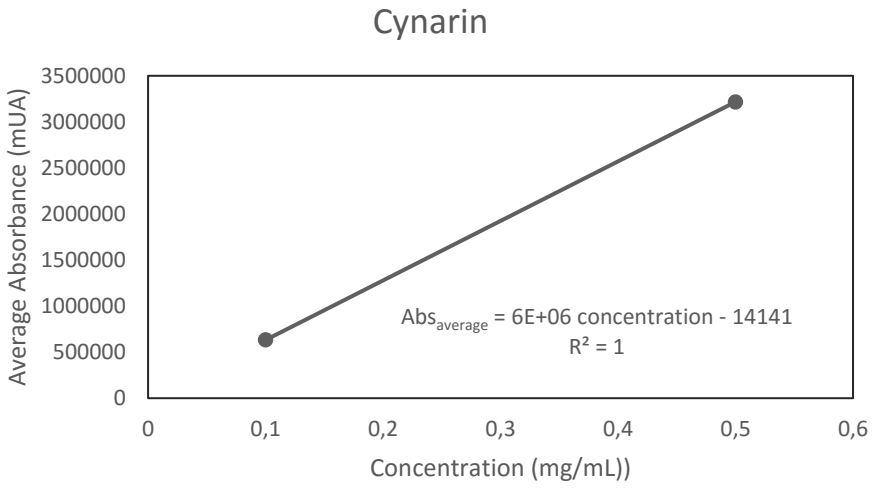
In both studies, *in vitro* and *in vivo*, the gel formulation presented the highest antioxidant activity, which can be explain by a higher release profile of the artichoke extract.

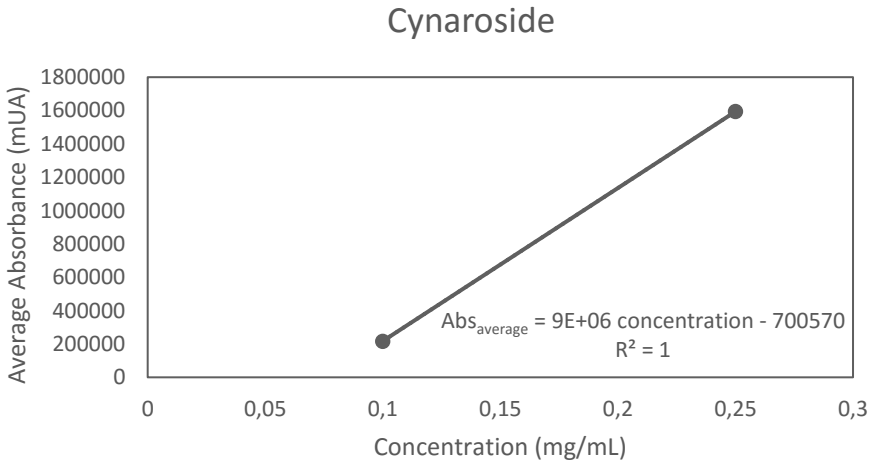
Formulations containing artichoke extract proved to be suitable, safe and efficient for skin application and for antioxidant purposes. Therefore, the main goal of this thesis was accomplished.

# 6. Chapter: Annexes

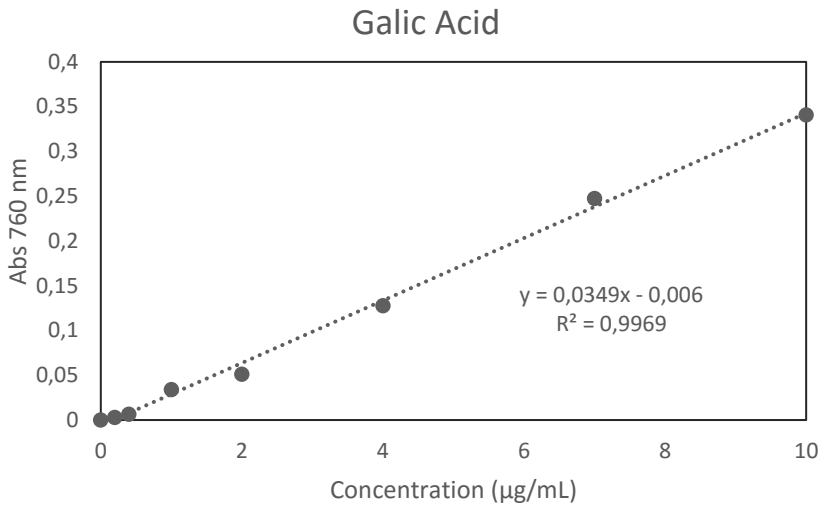
## 6.1. HPLC-DAD calibration curves for chemical characterization



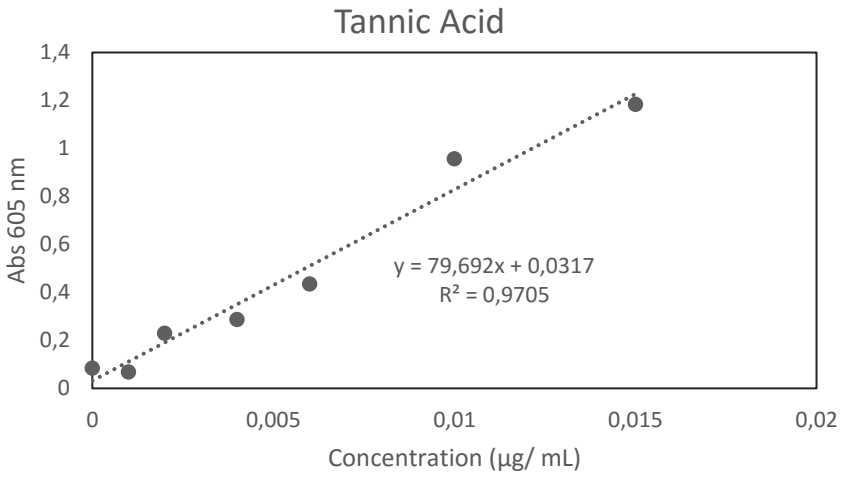



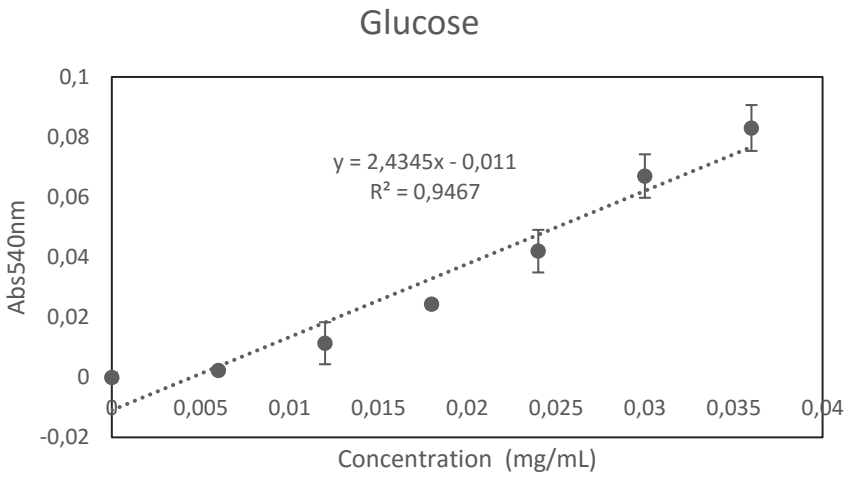
## 6.2. Folin-Ciocalteau test calibration curve



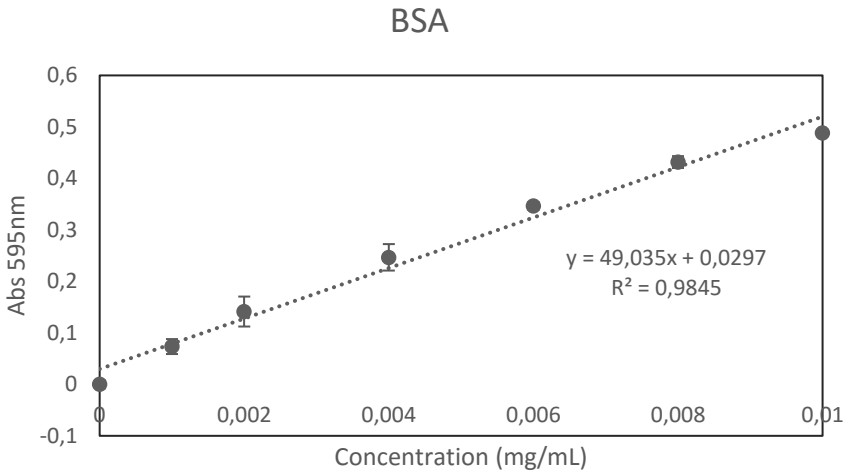

## 6.3. Prussian Blue test calibration curve



## 6.4. DNS test calibration curve




## 6.5. Bradford test calibration curve



## 6.6. Flavonoids test calibration curve